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The Ballistoscope: An Instrument for the Study of the Cardiac Impulse.*
(19759)

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A small container half-filled with a dark viscous fluid, such as commercial heavy oil, reflects the light of an otoscope bulb held at a distance above the container, in such a fashion that any swift movement of the surface upon which the container rests causes a definite and characteristic displacement of the reflected image. If the container is placed upon the human body, the motion of the image forms a pattern, the shape and size of which are determined by the motion at the point of support, and specifically by motion due to cardiac activity. Such an instrument, which can be used at the bedside as easily as a stethoscope, may be called a ballistoscope.

Description of ballistoscope. As seen from Fig. 1, the instrument has a heavy brass base, 2.5 cm high, with a diameter of 6.25 cm, upon which is soldered a container 5 cm high with a diameter of 3.75 cm. The thickness of its wall is 1.5 mm. It is partly filled with commercial heavy oil No. 40. An aluminum rod 20 cm high, fastened to the base, carries wires connecting a transformer or pair of dry cells with an otoscope bulb at the top of the rod. This supplies a current of 2.5 volts. The otoscope bulb is 18 cm above the oil surface within the container, and its reflection appears as a bright point on a black background.

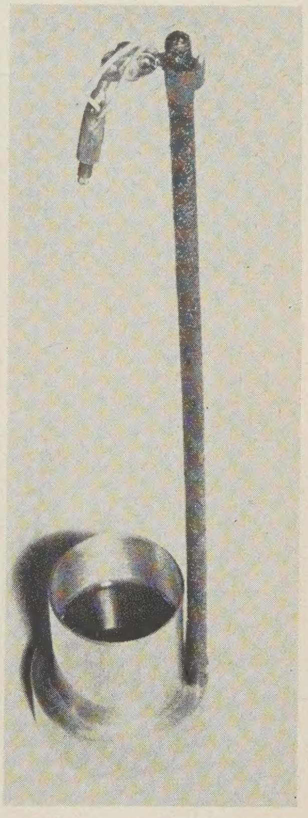

FIG. 1. Ballistoscope.

Procedure. The ballistoscope is placed

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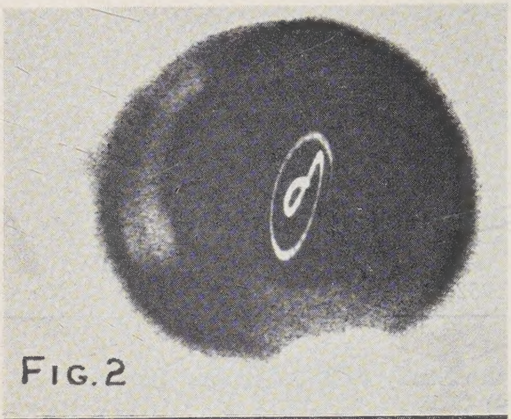

FIG.2

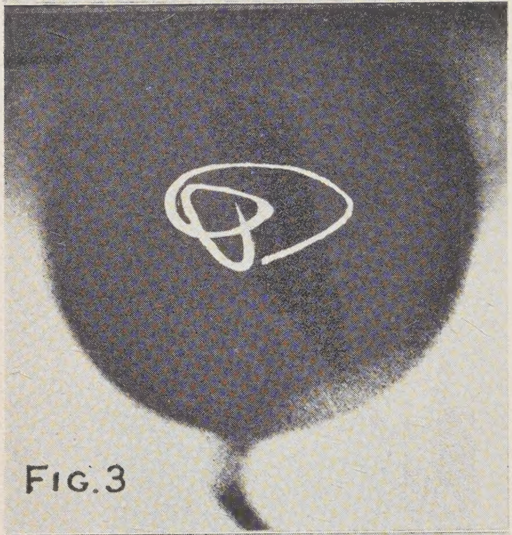

FIG.3

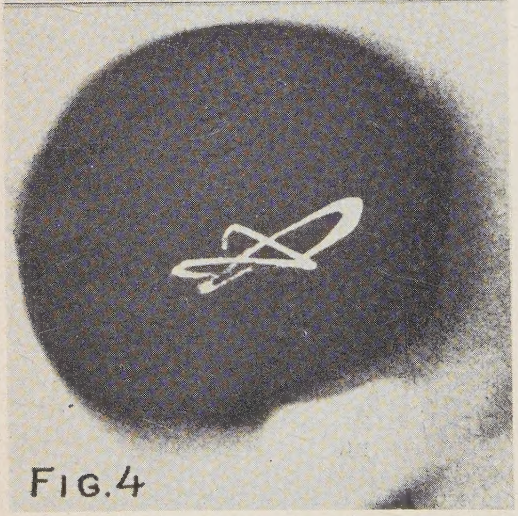

FIG.4

FIG. 2. Ballistoscope tracing of a normal individual.

FIG. 3. Ballistoscope tracing of a patient with mitral stenosis and insufficiency.

FIG. 4. Ballistoscope tracing of a patient with hypertensive cardiovascular disease and evidence of an old posterior wall infarction.

upon the lower third of the sternum with the patient in the supine position, so that the fluid surface is horizontal. The procedure can be employed in any desired position. If the patient is in a prone position, the ballistoscope is placed upon the back, and the reflection of the bulb is observed within the container. Similarly, the ballistoscope can be placed upon a bar across the lower legs, according to the procedure of Dock(1).

For the purpose of obtaining a permanent record, the above described procedure is followed, and a camera is focused upon the ballistoscopic flash within the container, which is photographed with a 0.5 second exposure. To identify the beginning and end of the tracing, and therefore its direction, the light of the otoscope bulb is switched on immediately after opening the shutter, causing the tracing to initiate as a thin thread, increasing steadily in thickness while the bulb is lighting up. When the shutter closes automatically, the light is interrupted suddenly, and the tracing ends therefore abruptly.

Results. As seen from Fig. 2 the ballistoscope tracing of a normal individual as obtained over the sternum is slightly elliptical, the long axis being in a longitudinal, and the short axis in a lateral, direction. The main portion of the tracing has a counterclockwise direction, and follows the first heart sound. It is succeeded by a short portion in a clockwise direction, which seems to follow the second heart sound when ballistoscopic observation is combined with auscultation. If the ballistoscope is placed upon a bar across the lower legs according to the Dock procedure, the configuration of the flash seems to duplicate the typical ballistocardiographic pattern.

Fig. 3 and 4 give examples of specific changes of the ballistoscopic pattern in patients with cardiac disease. Fig. 3, from a patient with mitral stenosis and insufficiency, is characterized by a clockwise direction of the tracing. The long axis is no longer longitudinal but more in a lateral direction. Fig. 4, from a patient with hypertensive cardiovascular disease and electrocardiographic evidence of an old posterior wall infarction, shows again a clockwise direction of its main portion, as well as a very pronounced lateral direction of its long axis. It is succeeded by a short swing in the opposite, namely counterclockwise, direction. The amplitude of both tracings (Fig. 3 and 4) appears larger than normal.

Discussion. The high viscosity of the fluid within the container produces a considerable damping effect, so that the relatively slow respiratory motion of the anterior chest wall does not produce any direct alteration of the configuration of the flash as caused by the forceful cardiac impulse. It produces, however, indirect alterations which can be interpreted as respiratory variations. The damping characteristics of the fluid are also responsible for the failure to register vibrations coincident with heart sounds or murmurs.

The mechanism of the ballistoscope seems to reside in the formation of a sloping reflecting surface during the ballistic movement of the underlying surface, such as the anterior chest wall. The degree of slope depends upon the magnitude of the cardiac impulse. Since the excursion of the reflex increases with the degree of slope, it will necessarily indicate the magnitude of the cardiac impulse provided the distance between object and mirror, in this case the distance between otoscope bulb and fluid surface within the container, is constant. If the distance between the bulb and fluid surface is increased, the excursion of the image will also increase.

Whereas the ballistocardiograph, in its usual form, is an instrument with one degree of freedom(2), namely, in a longitudinal direction only, the ballistoscope, as presented in this study, indicates movements in both longitudinal and lateral, as well as any other intermediate direction. It therefore can be considered an instrument with freedom in a two-dimensional plane, providing both factors of a vector, *i.e.*, direction and amplitude.

It has been pointed out that lateral ballistic movements may be of considerable importance (3,4). In view of its mechanical simplicity, the ballistoscope should lend itself particularly well for determining at a glance whether the main ballistic motion is longitudinal or lateral, and also furnish an idea about the amplitude. It can serve as a screening instrument at the bedside for further ballistocardiographic investigation.

Summary. An instrument called ballistoscope is described, which registers any motion of the underlying surface in a two-dimensional plane. Placed upon the sternum it reveals ballistic patterns which are constant and characteristic in normal individuals as well as in patients with various types of heart disease.

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Effect of Ethylenediamine upon Glycosuria of the Partially Depancreatized Rat. (19760)

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Ethylenediamine has been found to cause an increase in the amount of glucose excreted by the partially depancreatized rat during the period of its administration.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they were partially depancreatized(1) at a weight of 275 to 280 g. After diabetes was established all of the rats were placed in metabolism cages and force-fed a medium carbohydrate diet(2) by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technics and diet were modifications of those described by

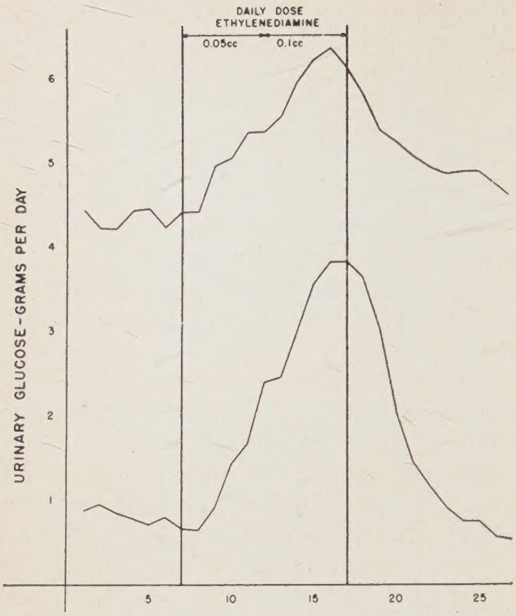

FIG. 1. Effect of ethylenediamine upon level of urinary glucose in partially depancreatized rats. Averages for 5 rats per group. Abscissa in days.

Reinecke, Ball and Samuels(3).

The animals were kept at a temperature of 74 to 78°F. Twenty-four hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with toluene. Urine glucose was determined by the method of Shaffer and Williams(4). Ethylenediamine, a liquid, was administered by subcutaneous injection.

Experiments and results. Five mildly diabetic rats and 5 moderately diabetic rats were each given 0.05 cc of ethylenediamine once daily for 5 days whereupon 0.1 cc was given daily in divided injections for 5 days. Each animal was observed for 10 days after the injections were stopped. As shown in Fig. 1, the glycosuria was intensified during the administration of ethylenediamine and then decreased to the preinjection level when the injections were stopped. This pattern of response was shown by each individual animal. Ethylenediamine caused a considerable amount of necrosis at the site of injection.

Discussion. Ethylenediamine is a toxic compound which is corrosive to tissue. It imposes a stress on the animal and was selected for study as a part of our research program on the metabolic consequences of various forms of stress. Other toxic agents causing skin damage, such as dilute solutions of formaldehyde(5), do not cause exacerbation of diabetes in the partially depancreatized rat. Apparently the diabetogenic effect of ethylenediamine is not characteristic of non-specific damaging agents. This effect is reversed when the compound is withdrawn. The mechanism of its action is not known to us.

Summary. The subcutaneous injection of ethylenediamine in 10 partially depancreatized rats caused exacerbation of diabetes in each animal but the glycosuria returned to the preinjection level when the administration of the compound was stopped.

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Penetration of Acetylcholine into Nerve Fibers Demonstrated by a New Chemical Method. (19761)

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In the course of pharmacodynamic studies on a number of quaternary ammonium compounds it was found that these compounds distributed themselves into the axoplasm of the giant axon of the Squid(1). In view of the existing controversy on the penetration of acetylcholine (ACh) into the interior of nerve fibers, distribution studies on ACh were carried out.

The affirmative evidence for the permeability of nerve fibers to ACh has been indirect. Lissak(2) showed that cholinergic axons, when stimulated, permit the outflow of ACh into the bathing Ringer's solution and Eccles(3) found that soaking the unanesthetized frog spinal cord in weak ACh solutions caused prolonged spontaneous discharges of the motor neurones. On the other hand, Nachmansohn(4) failed to demonstrate the presence of ACh labelled with N^{15} in the axoplasm extruded from Squid axons that had been immersed in 0.1 *M* ACh solution; it was not stated that the giant axons used were cleaned microscopically.

A sensitive chemical method for the determination of ACh(5) as well as improved techniques(6) for extruding uncontaminated axoplasm and for perfusing the axon sheath made it possible for us to demonstrate that ACh penetrates into the interior of the resting axon.

A chemical method for quaternary ammonium compounds has been adapted to the determination of ACh(5,7). To 0.5 cc of a sample containing ACh, 0.5 cc of bromphenol blue (0.08% in 30% K_2HPO_4) and 300 mg each of Na_2CO_3 and K_2HPO_4 were added. The colored dye complex formed was extracted by freshly washed ethylene dichloride (containing 4% iso-amyl alcohol). The ethylene dichloride extract was read in a spectrophotometer at 600 mμ. The lower limit of sensitivity is 1 μg per sample.

Two preparations were used: 1) axons prepared microscopically free of all adjacent connective tissue without injury to the axon; 2) axons prepared grossly free of excessive connective tissue without injury to the axon. In the first type of preparation, stainless steel needles were inserted into the cut ends of the axon and tied securely. The measured section of the axon, thus prepared, was immersed in ACh, 0.01 *M* in buffered artificial sea-water. After varying times of immersion, the axoplasm was extruded from the axon by forcing air and sea-water through the system, without injury to the axon sheath. In the second type of preparation, after immersion in 0.1 *M* ACh, the axoplasm was extruded by the conventional technic with adequate precautions against contamination. All experiments were carried out in the absence of any anticholinesterases. The results of the chemical determinations on the extruded axoplasm are summarized in Table I.

After extrusion of the axoplasm the axon sheath was filled with artificial buffered sea-water and immersed in 0.01 *M* ACh to determine the degree of penetration of ACh

TABLE I. Concentration of ACh in Axoplasm of Giant Axon.

Immersion time (min)	Molarity of ACh outside	Molarity* of ACh in axoplasm	Ratio, M inside / M outside	Type† of preparation
0	0	0	0	1
0	0	0	0	2
120	0	0	0	2
15	.01	.003	.3	1
30	.01	.0075	.75	1
20	.1	.009	.09	2
30	.1	.011	.11	2
60	.1	.023	.23	2
120	.1	.038	.38	2

* Calculated from the estimated volume of the section of axons used in preparation 1 and wt of axoplasm extruded in preparation 2 (calculations by both methods are in close agreement).

† 1—Microscopically cleaned fiber; 2—Grossly cleaned fiber.

through the sheath in the absence of axoplasm. The sea-water inside the axon was extruded after the immersion period and chemical determinations made. The rate of penetration of ACh was much more rapid in this case; equilibrium was reached between 5-10 minutes. At the end of the experiment, the axon sheath was tested for leaks by means of a water soluble dye.

In other experiments microscopically cleaned axons were immersed in 0.01 *M* ACh for 90 minutes and then washed for 10 seconds, 1 minute, and 30 minutes, in fresh aliquots of sea-water. The quantities of ACh determined in the washes were respectively: 4.4, 2.2, and 5.7 x 10^{-3} *M*. The 10 second wash was considered as surface contamination. The amount of ACh in the other washes was considered to represent a major portion of the ACh which had been distributed in the axon during the immersion period (molarities calculated from volume of axons).

Summary. The penetration of acetylcholine into the interior of the resting Squid axon, which had been immersed in ACh solutions, was demonstrated by its appearance in the extruded axoplasm. The outflow of ACh from resting axons previously immersed in ACh solutions was also demonstrated.

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Protein Separations and Interactions on Filter Paper.* (19762)

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Two technics are available whereby proteins may be caused to move on filter paper—chromatography and microelectrophoresis. The former method involves the movement of proteins by capillary ascent. A mixture of proteins diffuses from a spot into a streak by the action of an aqueous developing solvent (1). The latter technic, based on the Tiselius method, makes use of the different mobilities of the various proteins in an electric field(2). A combination of these two methods, chromatography in the first dimension followed by electrophoresis in the second dimension, provides a variation in the present 2-dimensional chromatography and makes it possible to study the separation and interaction of various proteins on filter paper.

Whatman No. 3 MM paper has been used throughout. The dry purified proteins are dissolved in 0.85% saline, and 0.04 ml aliquots are placed about 4 inches from the lower left hand corner of strips of filter paper approximately 8″ x 15″ in size. The spots are dried at room temperature, the papers formed into cylinders and stapled so that the edges do not touch, and then placed in shallow dishes containing the developing solvent. After the papers are thoroughly wetted by capillary action (about 1.5 hours), they are unstapled, rinsed quickly in veronal buffer solution (pH = 8.6), and placed in the electrophoresis apparatus.

The electrophoresis apparatus consists of a pair of plastic troughs (Perspex) 17″ x 4″ x 3¼″ each divided longitudinally into two compartments. Electrical contact between the

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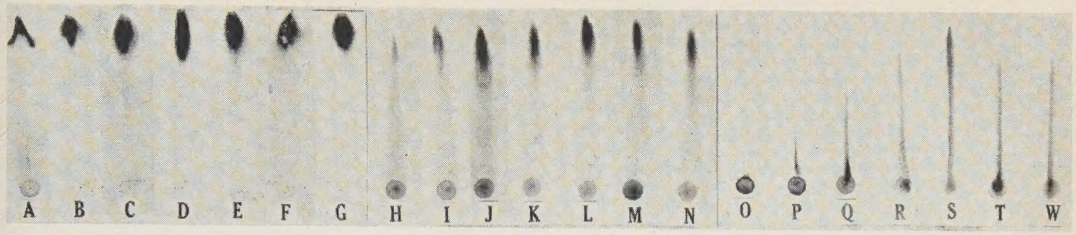


FIG. 1. Movement of proteins on filter paper at different hydrogen ion concentrations. A to G, .04 ml aliquots of a 3% solution of human serum albumin (Cohn Fraction V); H to N, .04 ml aliquots of a 2% solution of γ globulin (Cohn Fraction II); O to W, .04 ml aliquots of a 3% solution of fibrinogen (Cohn Fraction I). The following developing solvents were used: A, H, O, .05M potassium hydrogen phthalate pH 3; B, I, P, .05M potassium hydrogen phthalate pH 4.8; C, J, Q, .05M potassium hydrogen phthalate pH 5.4; D, K, R, .05M sodium citrate pH 6.1; E, L, S, .05M barbitone (veronal) pH 8.6; F, M, T, .1M sucrose unbuffered pH approximately 7; G, N, W, .1M sodium potassium tartrate unbuffered pH approximately 7.

buffer solutions in the outer and inner compartments is made through small wicks protruding through holes near the upper edge of the dividing wall. Carbon electrodes are placed in the outer compartments and connected through an ammeter and voltmeter to a source of DC from a selenium rectifier, with a variable AC input.

The filter paper strips are supported on a plastic frame fitted on to the troughs so that the ends of the papers dip into the buffer solutions in the two inner compartments of the troughs. The whole set-up is enclosed in a glass tank with a heavy glass lid resting on a rubber rim. Veronal buffer pH 8.6 consisting of 0.05 M sodium diethyl barbiturate and 0.01 M diethyl barbituric acid is used as the electrophoresis buffer solution. The filter paper strips are placed in such a position in the apparatus that the protein streak is about 3 inches from the surface of the liquid in the inner compartment of the negative electrode vessel.

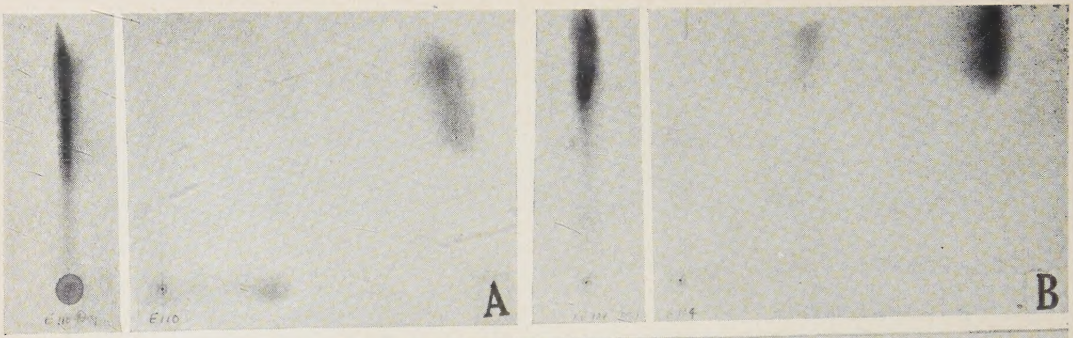
A potential of 130 volts is applied across the papers for about 18 hours, after which the papers are dried, and the proteins stained with hot aqueous solution of Solvay purple (0.05%) containing 0.5% sulphuric acid(3). After about 5 minutes the chromatograms are washed with warm water to remove the excess dye and dried.

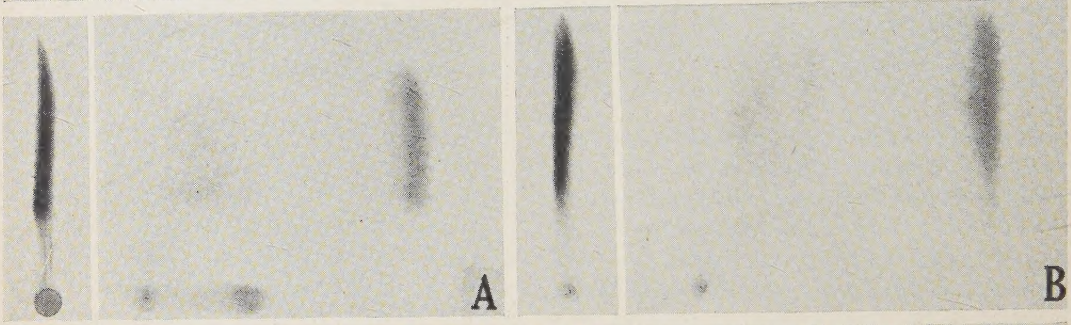
Effect of developing solvents at different hydrogen ion concentrations on movement of human serum albumin. The movement of albumin was investigated using 0.05 M phthalate buffer at pH 3, 4.8, 5.4, citrate buffer pH 6.1, veronal buffer pH 8.6, 0.1 M solution of sucrose and sodium potassium tartrate, both approximately pH 7. The position of albumin is virtually unchanged in all these developing solvents. All the albumin moves from the point of origin except with phthalate buffer pH 3. In this case some albumin remains at the origin. (Fig. 1, A to G.)

Effect of developing solvents on movement of γ globulin. Using the same developing solvents, γ globulin generally migrates to a position just below that of albumin, and a considerable portion remains fixed at the origin in all cases. At pH 3 in phthalate buffer, little protein moves up the paper as compared with that at pH 4.8, 5.4 etc. In veronal buffer pH 8.6, γ globulin moves to almost the same position as that of albumin. (Fig. 1, H to N).

Effect of developing solvents on movement of fibrinogen. In phthalate buffer pH 3 and 4.8, fibrinogen scarcely moves at all from the point of origin. At pH 5.4 there is a pronounced movement which becomes even more marked at pH 6.1 in citrate buffer. At pH 8.6 in veronal buffer, the movement is greatest, the fibrinogen flowing out from the point of application almost to the top of the paper, but with no well defined spot as is observed with albumin and γ globulin. The migrations in sucrose and sodium potassium tartrate solutions are similar to that in citrate buffer at pH 6.1 (Fig. 1, O to W).

Effect of developing solvents on the movement of albumin and fibrinogen using microelectrophoresis in the second dimension. In





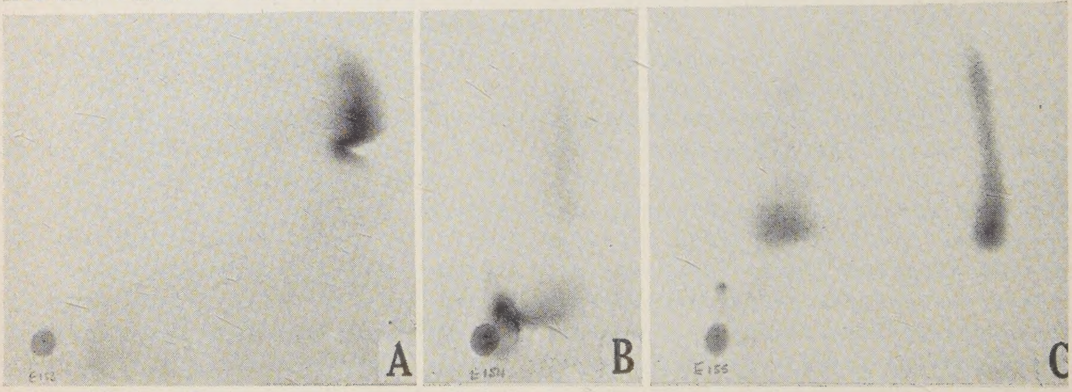


FIG. 2 (Top). Separation and interaction of albumin and fibrinogen on filter paper. A—.04 ml of a mixture containing 3% albumin and 3% fibrinogen chromatographed in the first dimension using phthalate buffer pH 4.8, showing the first dimensional streak, and the results of electrophoresis of this streak in the second dimension. B—Same as A, except chromatograph carried out in the first dimension using veronal buffer pH 8.6.

FIG. 3 (Middle). Separation and interaction of albumin, γ globulin and fibrinogen on filter paper. A—.04 ml of a mixture containing 3% albumin, 2% γ globulin and 3% fibrinogen chromatographed in the first dimension using phthalate buffer pH 4.8 showing the first dimensional streak and the results of electrophoresis in the second dimension. B—Same as A, except chromatograph carried out in the first dimension using veronal buffer pH 8.6.

FIG. 4 (Bottom). Effect of Tween 81 on movement of albumin and γ globulin using .1*M* sucrose solution in the first dimension and electrophoresis in the second dimension. A—.04 ml aliquot of a 3% solution of albumin containing .02 ml of Tween 81 per ml. B—.04 ml aliquot of a 3% solution of γ globulin containing .02 ml of Tween 81 per ml. C—.04 ml aliquot of a mixture containing 3% albumin, 3% γ globulin and .02 ml of Tween 81 per ml.

phthalate buffer at pH 4.8 fibrinogen, in contrast to albumin, hardly moves on the filter paper (Fig. 1, B and P). It should be possible, therefore, to separate albumin and fibrinogen by filter paper chromatography. When the two proteins are mixed and sent up the paper in phthalate buffer at pH 4.8, fibrinogen does not move, but albumin migrates as a more elongated band to a position below its usual one. The positions of these proteins are easily detected by electrophoresis (Fig. 2A). Thus fibrinogen exerts a marked effect on albumin apparently without itself migrating, *i.e.* some interaction seems to take place without sufficient combination occurring to be visible under the present experimental conditions.

At pH 5.4, fibrinogen alone migrates to some extent, but mixed with albumin, there is a separation into two components; the one remaining fixed at the point of origin and the other moving up in apparent combination with albumin.

This apparent combination, or interaction, is complete in citrate buffer at pH 6.1. All of the fibrinogen moves up from the starting point. Some uncombined albumin, however, must also be present because the albumin band extends beyond the fibrinogen band. This same effect is observed using veronal buffer pH 8.6, (Fig. 2B) or solutions of sucrose or sodium potassium tartrate. A trace of fibrinogen is found at the origin when tartrate is used as the developing solvent.

Effect of developing solvents on the movement of albumin, globulin and fibrinogen. A restrictive influence of fibrinogen on the movement of γ globulin occurs when a mixture of the two are chromatographed on filter paper. This restriction seems to disappear when albumin is added to the mixture. At pH 4.8, all of the albumin travels with γ globulin, the albumin band extending above that of γ globulin. The fibrinogen remains at the origin as before (Fig. 3A).

At pH 6.1 in citrate buffer all the proteins move up on the paper. Fibrinogen and γ globulin move together, both their bands being shorter than that of albumin. The phenomenon is observed at pH 8.6 in veronal buffer, (Fig. 3B) and in solutions of sucrose or sodium potassium tartrate.

Thus it would appear that the presence of fibrinogen alters the chromatographic positions of both albumin and of γ globulin, separately or together, a phenomenon which would be explained by mutual interaction between the proteins. When the three are mixed, γ globulin moves with albumin.

Effect of Tween 81 in the movement of albumin and γ globulin. The addition of surface active agents such as Tween 81 (polyethylene sorbitan monooleate) to solutions of albumin and γ globulin appears to alter the rate of movement of the proteins and causes elongation of the first dimensional streak. Compare Fig. 1 F and M with Fig. 4, A and B). This occurs when using the various buffer solutions or solutions of sucrose or sodium potassium tartrate as developing solvents. The albumin band is elongated, but the greater change occurs with γ globulin. Its band extends from the point of application to the top of the paper, with the largest portion appearing at the origin. When the two proteins are mixed and the same quantity of Tween 81 added, the albumin band is extended considerably and the γ globulin moves up with the albumin to a new position. No γ globulin appears at the origin, but most of the γ globulin still appears in the lower part of the first dimensional streak.

The phenomena are consistent with the conclusion(1) that complex formation between the proteins and the surface active agent occurs, as well as interaction between the proteins themselves.

Conclusion. Using a combination of two technics, paper chromatography in the first dimension and paper microelectrophoresis in the second dimension, it is shown that protein separation may take place during capillary ascent of a mixture of proteins on filter paper and that protein interaction may also occur.

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Infra Red Absorption Spectra of Viruses. (19763)

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Infra red (IR) spectrometry has been of use primarily in the analysis of organic compounds and is thus being applied extensively as a quantitative and qualitative method(1). Within recent years IR spectrometry has been used in the study of biological problems, as exemplified in the study of urinary ketosteroids(2), porphyrins(3) and dextran(4). Reports(5,6) indicate that bacteria exhibit different IR absorption spectra which may eventually provide a means for their rapid identification.

Since viral agents may be less complex in their chemical constitution than bacteria, and perhaps thereby provide absorption spectra of more distinctive nature, such agents have been processed to relative purity and examined for their IR absorption characteristics. This type of examination might provide a rapid method of viral identification and delineation of their interrelationships.

Methods. Viruses, as follows, were processed and examined for their IR absorption spectra: meningopneumonitis (MP) (Francis strain),* ornithosis (P-4 strain),* mumps (Enders strain)* and Newcastle disease virus (NDV) (California strain).†

Embryonated chicken eggs were inoculated into the chorio-allantoic cavity with 0.1 cc of 10^{-1} dilution of the viruses noted above. Ornithosis and meningopneumonitis agents were inoculated into 9-day-old embryos and chorio-allantoic fluids (CAF) were harvested 3 days later from the dead embryos. NDV was inoculated into 10-day-old embryos and CAF were harvested from the dead embryos 2 days later. Mumps virus was inoculated into 7-day-old embryos and CAF were collected 5 days later. In this manner, all of the CAF specimens came from 12-day-old incubating embryos. The fluids were each centrifuged at 3000 rpm for 30 minutes and the clear supernates were then centrifuged at 40,000 rpm‡ (140,000 x G) for one hour. The sedimented pellets were then resuspended in double distilled water to 1/10 of the original CAF volume. The slightly turbid suspensions were tested for concentration by microscopic examination of the MP and ornithosis viruses and by hemagglutination titration of the NDV and mumps viruses. All of the virus preparations were sealed in glass ampoules and stored in the CO_2 box.

In preparing specimens for IR spectrometry, the virus suspensions were spread evenly with a rubber policeman over the surface of Ag S-coated silver chloride plates§ (1 inch square x 1 mm thick). Approximately 0.2 ml was applied to each plate. The specimen was then desiccated in a 37°C dry incubator and stored overnight in a desiccator jar containing "drierite." Each dry specimen was then covered with a blank AgCl plate and both were sealed together at the edges with plastic ("Scotch") tape. The plates were then wrapped in paper and stored with "drierite" until examined. Two different lots of each virus, in duplicate, were examined.

The IR analyses were determined in a Baird Model B, double beam, recording infra-red spectrophotometer. The silver chloride slides were mounted in fiberboard carriers with an aperture of 13 mm. Carrier dimensions were approximately 7 x 15 cm. The blank was composed of silver chloride sheets sandwiched together as in the preparation of the specimens and mounted on a similar carrier.

Results. Fig. 1 demonstrates the IR absorption spectra obtained with the 4 viruses noted above. Between the wave lengths of 8 to 12 μ, the curves of mumps and MP viruses appear

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* Obtained from the Viral and Rickettsial Registry of the American Type Culture Collection.

† Obtained from Dr. Raymond Bankowski, School of Veterinary Medicine, University of California, Davis.

‡ Spinco Ultracentrifuge, Specialized Instruments Co., Belmont, Calif.

§ Purchased from the Harshaw Chemical Co., Cleveland, Ohio.

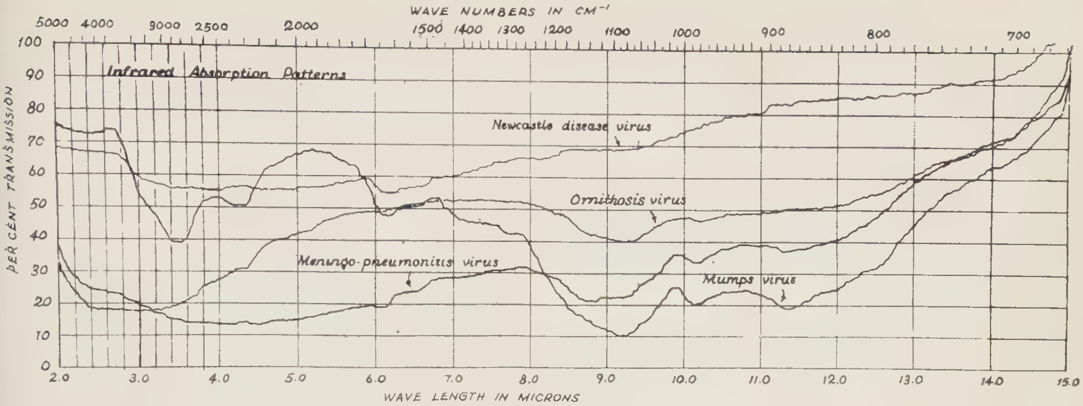

FIG. 1.

to be similar. Between these two points ornithosis virus tends to resemble the above two viruses, whereas NDV is entirely different. Between the wave lengths of 3 and 8 μ, mumps virus demonstrates an absorption pattern which is distinctly different from that of the 3 other agents. MP and ornithosis viruses have some slight similarity between these points. The NDV curve shows a slight dip between 6 and 7 μ, otherwise, it bears little resemblance to any of the other 3 viruses. All of the preparations of each virus examined were essentially the same.

Discussion. In general, there appears to be some similarity between the absorption spectra of MP and ornithosis viruses, whereas the spectra of mumps and of NDV viruses can be distinctly differentiated. MP and ornithosis viruses are similar morphologically and possess antigenic properties which are difficult to differentiate. This close relationship may explain the similarity of the IR absorption spectra demonstrated for these two agents. Mumps virus has no detectable morphological or antigenic relationship to MP and ornithosis viruses and appears to be distinctly different from the latter two in its IR absorption spectrum. NDV is unrelated to the other 3 viruses in morphology, in antigenic properties and apparently in its IR absorption spectrum (Fig. 1).

Since all of the CAF specimens were collected from embryos of the same age, this factor might be ruled out as an explanation for the divergence of absorption spectra. The fact that mumps virus was collected from live embryos, whereas the other three came from dead embryos, might introduce a significant variant that should warrant further study.

The curves depicted in Fig. 1 may not be definitive tracings since the viral preparations were still relatively crude. More precisely refined agents may produce IR absorption spectra of analytical value. From evidence thus far examined, this method of studying viral agents may be useful eventually both for their identification and for their classification.

Summary. Infra red absorption spectra have been determined of meningopneumonitis, ornithosis, mumps, and Newcastle disease viruses. The spectra of mumps and Newcastle disease are each distinctly different from that of meningopneumonitis and ornithosis, whereas the spectra of the latter two agents seem to indicate a close interrelationship.

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Exogenous Emulsifiers and Fat Absorption.* (19764)

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The rather general acceptance in the recent past of Frazer's(1) partition hypothesis seems to have strengthened the idea that emulsifying agents should hasten the rate of the absorption of fat from the intestine. Such an effect might be the result of a greater emulsification which may either promote the speed of absorption of the finely divided particulate fat or permit a more rapid action of the lipolytic enzymes.

The findings of several investigators could be interpreted as evidence supporting this effect of emulsifiers. An increased rate of fat absorption has been obtained when the ingested fat is supplemented with lecithin(2-4), which has an emulsifying action. Becker *et al.*(5) reported Tween 80 to affect fat absorption differently in young and aged people, but their results seemed to indicate a faster rate of fat absorption in both groups. Tween 80 has been found by Jones *et al.*(6) to promote the absorption of fat and fat soluble substances by patients with steatorrhea. However, they stated that in the normal subject "the absorption of dietary fat is so nearly complete that the addition of such an agent to the diet would seem to be of negligible value." An increased rate of fat absorption has also been reported to occur when an emulsified fat is instilled in the ligated rat intestine.

Earlier studies of Holt *et al.*(8) gave no evidence that the normal infant found any difficulty in intestinal emulsification of dietary fat. The results of Jones *et al.* were not confirmed by Badenoch, Johnson and their collaborators(9) and the latter group reported Tween 20 to be ineffective in reducing the fecal fat of prematures and of infants with steatorrhea. Recently, Shoshkes *et al.*(10) found absorption of fat to be unaffected by the presence of 20% lecithin or Tween 80, amounts greatly in excess of that required to make excellent emulsions by mechanical means.

With this somewhat conflicting evidence, it seemed wise to include some of the synthetic emulsifiers in our studies of the various factors influencing the rate of fat absorption. The effect of a monoglyceride† and of a number of the fatty acid esters of some sorbitol or alkylene oxide derivatives‡ on this rate has been determined at different levels of fat intake and emulsifying supplement. Several of the latter emulsifying agents were included in these studies because they have slightly different physical properties. Also an attempt has been made to learn whether these substances influenced either gastrointestinal motility or fat splitting, since such actions might affect the rate of fat absorption.

Experimental. The groups of animals used were all male or female albino rats with weights ranging from 150 to 500 g. As checks upon each other, two methods, the chylomicrograph and a modified Cori technic, were used to determine the rate of fat absorption.

A number of investigators(4,5,11,12) have shown the former method can be used to determine the relative amount of fat in the blood and that the area under the curve obtained gives a similar measure of the rate of the various processes involved. After a 24-hour fast, the rats were given 0.05 ml of olive oil per square decimeter of body surface with or without the supplement of 6 or 20% of the emulsifier. The small amount of fat permit-

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* The funds for this study were provided in part through the courtesy of The Atlas Powder Co., Wilmington, Del.

† Generously supplied by Distillation Products, Inc., Rochester, N. Y.

‡ Atlas Powder Company trade marks and chemical descriptions:

Tween 80—polyoxyethylene (20) sorbitan monooleate

Myrj 45—polyoxyethylene (8) stearate

Span 60—sorbitan monostearate

Tween 60—polyoxyethylene (20) sorbitan monostearate

TABLE I. Effect of Supplements of Various Emulsifiers on Blood Fat Levels after Fat Ingestion (Chylomicrographs).

No. rats	Fat supplement	Chylomicron count: Fasting	Hr: 1	1½	2	3	Area under curve	"t" value
8	Saline	13	36	41	42	44	83 ± 6	
10	Tween 80 6%	15	41	47	46	45	91 ± 3	1.285
8	" 20%	8	47	44	51	53	98 ± 6	1.696
10	Saline	6	69	43	44	40	92 ± 5	
	Myrj 45 6%	4	58	51	49	36	95 ± 5	.392
	Span 60 6%	4	63	48	51	49	102 ± 8	1.184
	Tween 60 6%	5	64	46	45	55	100 ± 5	1.223
	Monooleate 6%	4	66	55	41	44	97 ± 8	.538
35	Saline	8	48	52	52	44	99 ± 4	

Including the stand. error of the mean and Fisher's "t" value for areas under the curve.

ted a more accurate counting of the chylomicrons and the use of a more physiological intake of fat. The supplements given approximated the levels used by Jones, Becker, and Stare in their studies. The method followed was that previously used in this laboratory(4). An eyepiece micrometer disc (5 mm square divided into 100 squares) replaced the Whipple disc, and from the 4 or more fields of 100 squares counted, the average number of chylomicrons in 10 squares were the counts recorded.

The second method of this study made use of the Cori technic as modified by Deuel *et al.*(13). The determination of the rate of fat absorption by a recovery of unabsorbed fat after an interval for absorption has been found satisfactory by a number of investigators(7,14). The ages or sex of the animals did not influence this absorption rate. The rats were fasted 48 hours and given by tube 0.15 or 0.30 ml of Sudan IV stained olive oil per square decimeter of body surface. After a 2- or 3-hour period for absorption the rats were sacrificed and the distance the stained fat had passed along the intestine as well as the total intestinal length measured. Finally, the fat was recovered, weighed and the free fatty acids titrated. This procedure permitted not only an estimation of the per cent of fat absorbed and the rate of this process, but also a relative measure of gastrointestinal motility and fat splitting as affected by the various supplements given.

Results and discussion. The chylomicrographs, obtained in 2 experiments when the fat was given alone or with either 6 or 20% of the exogenous emulsifiers, are shown in Table I. Although the area under the curve for the first group receiving only the fat appears to be much smaller than that of those receiving the 20 or 6% of Tween 80, the differences are not statistically significant. Wide individual differences in this particular control group (fat alone), and a mean control value of 99 on 35 rats involved in all studies about this time, would indicate that the differences should not be significant. A later experiment confirmed this conclusion. That similar results were obtained when supplements of 6% Myrj 45, Span 60, Tween 60 and glyceryl monooleate were given with the fat may be seen in Table I. The similar peaks and areas under the curves indicated comparable fat absorption in all groups. Hence, the use of this method gave no evidence of any change in the rate of the absorption of the fat when it was supplemented with any of the emulsifiers.

The results obtained by the modified Cori technic are included in Table II. In the first experiment, there was no significant change in the per cent of fat absorbed or in the rate of its absorption over a 2-hour period when 0.15 ml of fat per square decimeter of body surface was supplemented with 20% Tween 80 or glyceryl monooleate. Similar results were obtained when twice as much fat was supplemented with 6% of the previously mentioned exogenous emulsifiers and the absorptive period extended to 3 hours. The latter changes permitted a comparison of our values with those of Deuel and co-workers when they fed cottonseed oil. Good agreement was ob-

TABLE II. Absorption and Splitting of Fats, and Intestinal Motility as Affected by Various Supplements of Emulsifiers (by Recovery).

No. rats	Fat supplement	Fat absorbed		"t" value	% of free fatty acids recovered	% of length of intestine traversed
		%	mg/dm²/hr			
16*	Saline	33.9	21.6 ± 1.7		17.5	80
8*	Tween 80 20%	32	22.9 ± 1.6	1.067	—	90
8*	Monooleate 20%	36.8	25.6 ± 2.5	2.068	16.5	81
8	Saline	44.3	40.3 ± 2.9		17	85
8	Myrj 45	47.8	43.9 ± 3	.860	15.9	94
9	Span 60 6%	48.8	44.6 ± 1	1.263	12	85
7	Tween 60	42.3	39 ± 3.6	.284	12.5	92

* 2 hr absorptive period after ingestion of .15 ml of fat per dm² of body surface; all others, 3 hr and .30 ml of fat.

served. Again, there was no evidence of an effect upon the rate of fat absorption when it was determined by the recovery method instead of from changes in the blood fat levels.

The amounts of free fatty acids in the recovered fat and the relative length of the intestine traversed appear to be unaffected by the presence of these amounts of the emulsifiers. These results are in agreement with previous, and as yet unpublished, data from similar tests. Thus, the evidence suggests no change in the fat splitting or intestinal motility in the animals given the fat and supplement, as compared with a control group receiving the fat alone.

It appears important that it be established whether the physical effect of an increased emulsification of the fat in the intestine of the normal animal plays a role in promoting fat absorption. Our results from the use of 2 methods are in complete agreement with those from Stare's laboratory—that the absorption of orally fed fat is unaffected by the presence of emulsifiers, even when greatly in excess over that needed to make excellent emulsions by mechanical means. Factors, such as splitting and fat passage along the intestine, that might affect such absorption also appear unchanged. It seems logical to suppose that under normal conditions, a sufficient emulsification of the fat would naturally occur in the normal intestine to permit the maximum absorption of the fat. If this be true, then use of exogenous emulsifiers as fat supplements should not affect the rate of fat absorption in the normal subject. Such reasoning is in accord with our findings.

If it be established that emulsifier supplements do not promote fat absorption, then the effect of lecithin must be otherwise explained. The fact that both lecithin and choline(4) appear to increase the rate of fat absorption would suggest that one or both are involved in some chemical mechanism required for fat absorption. Thus it appears that the presence of substances that may promote changes in the physical state of the fat do not increase the rate of the absorptive process in the normal animal, while those that may enter into the chemical actions involved do promote fat absorption.

Summary. As determined by changes in the blood fat levels and by recovery of fat from the intestine after an absorptive period, the rate of fat absorption in normal, orally fed rats appears to be unaffected by 6 or 20% supplements of a number of exogenous emulsifiers. The lack of changes in the degree of fat splitting and intestinal motility which could affect fat absorption are in complete agreement with the above finding. The action of lecithin in promoting fat absorption seems best explained by other than its emulsifying action.

The technical assistance of Mrs. Anne M. Miller is acknowledged.

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Effects of Single Intravenous Injections of Pituitary Growth Hormone to Normal Adult Men.* (19765)

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Highly purified preparations of pituitary growth hormone† are capable of inducing consistent effects in laboratory animals. Thus growth hormone accelerates the growth rate of both hypophysectomized and normal animals(1); induces a permanent state of diabetes mellitus in certain species(2),‡ increases the ketosis of the fasting state(3); decreases total body fat(4); and diminishes the urinary excretion of sodium and potassium(5). In contrast this hormone has, in general, been ineffective in man. Recently, however, positive nitrogen and phosphorus balance has been reported with growth hormone in a case of hypopituitarism(7), and hyperglycemia of short duration has been observed in a patient with pancreatic islet cell tumor(8).

It is the purpose of this paper to report briefly experiments which demonstrate consistent, rapidly occurring effects of a highly purified growth hormone preparation§ in man.

Methods. Eight subjects were studied, 7 normal men (ages 20-29) who were ambulatory except during the experiment, and a 42-year-old patient with cancer of the stomach and malnutrition. Five of the normal subjects were admitted to the Metabolic Ward of the Royal Victoria Hospital for 4 days. Dietary intakes were not controlled before or during the course of the experiments. On day 1 no food was taken after 6 p.m. The bladder was emptied at midnight and 500 cc of water administered orally. On day 2 urines were collected at 6 a.m. and hourly thereafter until 1 or 2 p.m. Amino acid nitrogen(11), glucose, phosphorus and creatinine (Evelyn modification of Folin-Wu) determinations were done on these specimens. 250 cc of water were given at 6, 7, 10 and 11 a.m. and 12 noon to insure adequate urine volumes but the fast was continued until the last urine was collected. Beginning at 8:15 a.m. on day 2, 320 cc of an amino acid solution (1.24% amino N) was administered intravenously at a constant rate over a period of 26 minutes. Venous blood samples were drawn without stasis and the following determinations done: amino acid

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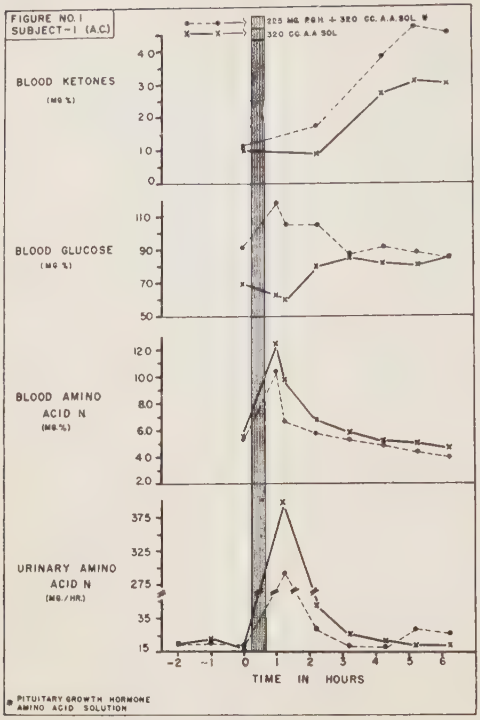
* The expense of these studies was partially defrayed by the National Research Council, Ottawa, the Damon Runyon Memorial Fund, and Frank W. Horner Ltd., Montreal.

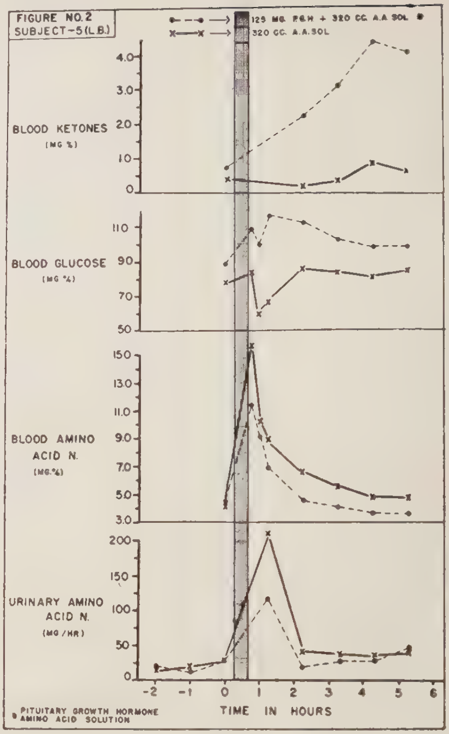
† Hereafter referred to as growth hormone.

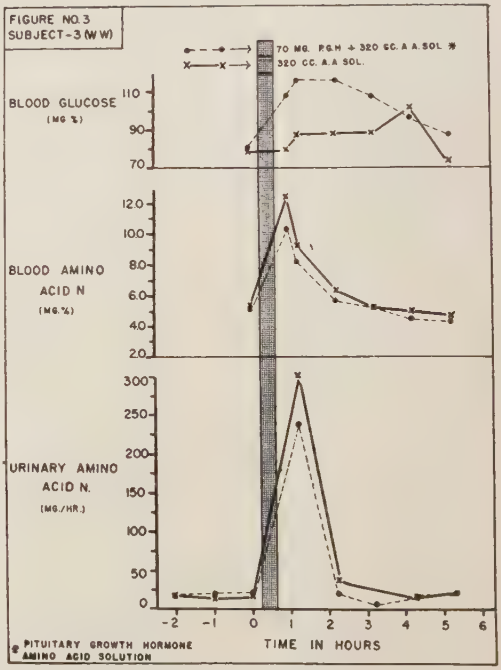
‡ Raben and Westermeyer(6) reported that they had obtained a purified growth hormone preparation which did not exhibit diabetogenic properties in 3 dogs.

§ Generously supplied by Dr. Leonard Mitchell of Frank W. Horner Ltd., Montreal. This preparation was isolated from beef pituitaries by the Wilhelmi method(9). Its biological activity is equal to that of the electrophoretically homogeneous preparation of Li(10). It is very low in ACTH, TSH, gonadotropin and pitressin activity by biologic assay.

‖ Kindly supplied by Dr. J. H. Laurie of Merck Co. Ltd., Montreal. This solution contains 12 amino acids; 10 are the l-form, 1 the d-l (tryptophane).







nitrogen(12), glucose (Folin-Wu), non-protein nitrogen (Evelyn modification of Folin-Wu), ketones(13), eosinophiles, phosphorus (Fiske-Subbarow) and alkaline phosphatase (King-Armstrong). Amino acid nitrogen, glucose, ketones, eosinophiles were determined on heparinized blood. Day 3 was a rest period. On day 4 the injection of amino acids was repeated with the addition of growth hormone (through the rubber tubing of the I.V. set) at a constant rate over the entire injection period. Three dose levels of growth hormone were used: 1, 2 and 3 mg/kg body weight. In two normal subjects the amino acid solution was administered the second time without growth hormone. In the patient with cancer the order of the experiment was reversed, that is the hormone given on day 2 and 250 cc of amino acid solution administered over a period of 30 minutes instead of 320 cc over a period of 26 minutes.

Results. Fig. 1, 2 and 3 illustrate the changes observed in blood and urinary amino acid nitrogen levels, blood glucose and blood ketones in 3 cases at the various dose levels. The principal changes in blood and urinary amino acid nitrogen in seven experiments are presented in Tables I and II. In the control experiments the blood amino acid nitrogen

TABLE I. Blood and Urinary Amino Acid Nitrogen Data.

Subject	Dose of PGH	Blood amino acid nitrogen (mg %) Fasting Control	Fasting PGH	Maximum* Control	Maximum* PGH	Diff.	% diff.	Total urinary amino acid nitrogen (mg) Control	PGH	Diff.	% diff.
1–A.C.	225 mg (3 mg/kg)	5.44	5.30	12.52	10.40	1.98	29	517.1	406.2	110.9	21
2–R.B.	200 mg (3 mg/kg)	5	4.80	11.54	9.24	2.10	32	340.3	252	88.3	25
3–W.W.	70 mg (1 mg/kg)	5.28	5.92	12.52	10.50	2.66	36	377.9	292.3	85.6	23
4–D.K.	125 mg (2 mg/kg)	4.80	4.88	16.16	13	3.24	28	332.2	254.2	78	23
5–L.B.	125 mg (2 mg/kg)	4.48	4.72	15.38	11.52	4.10	37	332.1	232.1	100	30
7–M.I.†	92 mg (2 mg/kg)	4.88	4.72	18.04	13.88	4.16	31	372.5	198.7	173.8	47
8–V.S.‡	0	5.20 5.26		12.04 11.50		.60	8	245.8 279		33.2	11

* In subjects 1, 2 and 3 these samples were taken 15 min after the intrav. infusion was ended, in the others 5 min. after the end of the infusion.
† Subject with cancer of the stomach and malnutrition. ‡ Subject who received amino acid solution alone twice.

TABLE II. Time Required for Blood Amino Acid Nitrogen Levels to Return to Fasting Level after Start of the Amino Acid Infusion (in Hr).

Subject	Control	Growth hormone
1	4	3
2	3	2
3	3	2
4	3	1
5	5	2
7	5	3
8 (2 controls)	3 3	—

values rose to a maximum immediately after the cessation of the amino acid infusion, returned to fasting levels within 3 to 5 hours and remained approximately at this level, the terminal values falling slightly in two cases. With growth hormone the maximum values were reached at the same time but were consistently lower than the controls by 1.98 to 4.16 mg %, which represents an average of 32% (range 28-37%) less than the control values, and they remained below control figures throughout. The values returned to fasting levels within 1 to 3 hours as compared with 3-5 hours in the controls, and subsequently fell below the fasting values, remaining so for the duration of the experiment. Blood non-protein nitrogen minus amino acid nitrogen values were not increased in the growth hormone experiments (Fig. 4).

The total excretion of amino acid nitrogen from 8 a.m. to the end of the experiment was 68-111 mg less under the influence of growth hormone. This represents an average reduction of 24% (range 21-30%) in relation to control values.

The blood glucose levels during the first part of the experiment were consistently elevated under the influence of growth hormone, whereas in the controls a depression of the fasting level usually resulted at this time. Glycosuria was not observed under the influence of growth hormone.

In the 3 cases in which blood ketones were studied, the ketonemia of fasting was accentuated by growth hormone: in subject 1 the increase in the maximum blood ketone value was from 3.18 to 4.80 mg %, in subject 4 from 2.81 to 3.75 mg %, and in subject 5

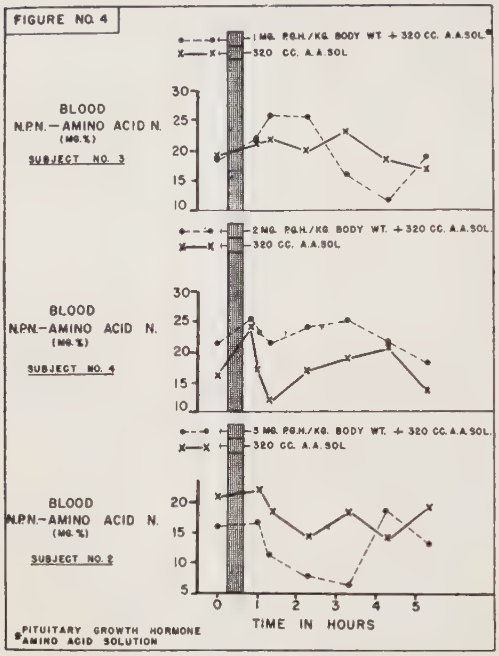


FIGURE NO. 4

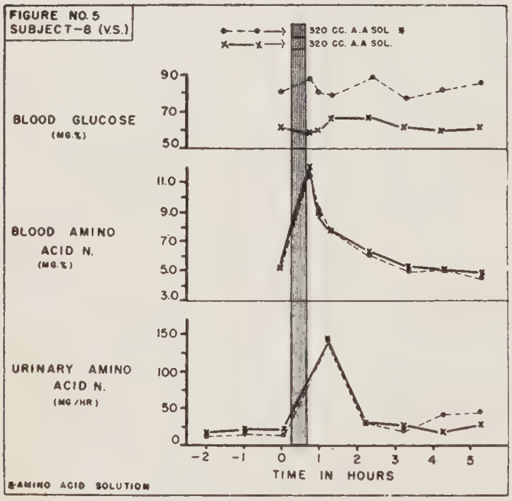


FIGURE NO. 5
SUBJECT—8 (V.S.)

from 0.87 to 4.39 mg % in the last 2 hours of the experiment. Serum and urinary phosphorus and serum alkaline phosphatase showed no consistent variation from control values. In all but 2 cases (those receiving 3 mg/kg) blood eosinophiles remained the same or showed the usual diurnal variation. With the 3 mg/kg dose of growth hormone there was a depression below control curves. Blood hematocrit values in 2 cases so studied were not significantly altered.

Of the 3 subjects tested, No. 3 and 4 showed a marked increase in the urinary sodium retaining activity (method devised by Miss Bertha Singer, based on the retention of radioactive sodium in adrenalectomized rats); in the third subject (No. 2) the assay was unsatisfactory.

In the patient with malnutrition and cancer, reversing the order of the experiment did not qualitatively alter the amino acid nitrogen results (Fig. 6). With growth hormone the maximum blood amino acid nitrogen value was 30% less and the total amino acid nitrogen excretion in the urine 47% less than the control values. The maximum blood ketone value with growth hormone was 1.33 mg % higher than the corresponding control figure. The glucose curves could not be interpreted because the patient had a diabetic glucose tolerance curve.

In order to determine whether the prior administration of the amino acid solution influenced the effect of the subsequent infusion, 2 amino acid infusions alone were administered to two normal subjects on days 2 and 4. In one the maximum blood amino acid nitrogen value was 8% less in the second experiment but subsequent values were either slightly above or below the corresponding ones in the first (Fig. 5). The total quantity of amino acid nitrogen excreted in the urine during the second experiment was 11% higher than during the first. The curves were similar in the second control.

The 2 subjects receiving the largest dose (3 mg/kg body weight) had nausea and vomited a small amount. The amount of amino acid nitrogen in the vomitus was negligible. Two subjects receiving 2 mg/kg body weight complained of a mild, transient headache. The remaining 2 subjects had no symptoms. None of the subjects developed pyrexia during the experiments. No significant changes in blood pressure or pulse rate were observed.

Discussion. The data reveal that growth hormone given intravenously has a rapid effect (within 5 minutes after the end of the infusion) on blood amino acid nitrogen and glucose levels. The effect on the former lasted

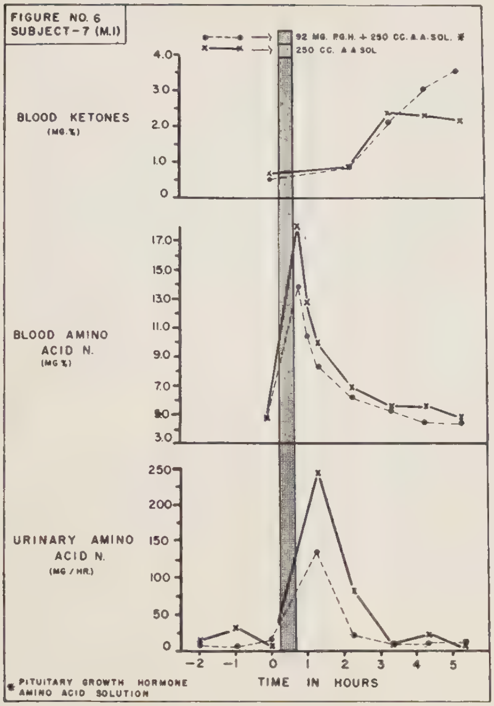


FIGURE NO. 6
SUBJECT-7 (M.I)

at least 6 hours, whereas the glucose effect disappeared in one to 2 hours. These effects were observed with doses of growth hormone ranging from 70 to 225 mg per subject. The amino acid nitrogen changes in blood and urine without increase in non-protein nitrogen probably means a significant retention of the administered amino acids somewhere in the body—presumably the intracellular and/or extracellular space. The results might also be explained by an increase in blood volume, an increased catabolism of the administered amino acids, or an expansion of the extracellular space. The fact that blood hematocrit values were unchanged by growth hormone (two cases studied) and the non-protein nitrogen not increased (3 cases studied) is against the first two possibilities. It is planned to do measurements of extracellular space to rule out the third explanation. Finally, the possibility remains that an increased rate of anabolism took place under the influence of growth hormone.

In the rat growth hormone depresses the fasting level of amino acid nitrogen(14), increases the rate of removal of amino acids from the plasma when protein hydrolysate is administered(15), and diminishes the formation of urea from administered protein hydrolysate(16). In the rat, hypoglycemia is observed following growth hormone administration(17), whether or not protein hydrolysate is given(15). However, in these experiments the blood glucose determinations were done on samples taken 1½ to 2 hours after growth hormone was administered intraperitoneally. In our experiments the hyperglycemia occurred during the first 1½ hours after the end of the intravenous infusion of growth hormone. Failure to obtain the serum phosphorus(18) and alkaline phosphatase(19) changes which occur in the rat in more prolonged experiments is not surprising owing to the short duration of the experiments.

The failure of others to demonstrate positive effects in man with large doses of growth hormone may be due to the fact that it was given subcutaneously or intramuscularly. It may be that the hormone is rapidly inactivated by subcutaneous and muscle tissue. Another possible explanation is the rapid formation of antibodies with repeated doses, although no such antibody formation has been demonstrated in man(20) or in the rat(21). Studies on antibody formation after intravenous growth hormone administration are being done.

Summary. A highly purified preparation of pituitary growth hormone given intravenously, together with an infusion of amino acids, to 5 normal young adult men and a patient with cancer caused a consistent, rapid, sustained lowering of blood amino acid nitrogen levels and of amino acid nitrogen excretion in the urine; a rapidly occurring but transient hyperglycemia; an increase in the ketonemia of the fasting state in three subjects so studied and an increased urinary excretion of sodium-retaining material in the 2 subjects so tested. No consistent effects were noted on blood and urine phosphorus, serum alkaline phosphatase or blood eosinophiles.

We wish to thank Dr. Norman Kalant for permission to use his ketone determinations and Miss Bertha Singer for permitting us to use the sodium retention assay data. Thanks are due to Miss Lois

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Effect of Nucleic Acid Derivatives in the Reversal of Aminopterin Inhibition in the Chick Embryo.* (19766)

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Aminopterin (4-amino-pteroylglutamic acid) is a powerful inhibitor of embryonic development in the chick(1,2). The injection of 10 μg or more into eggs prior to incubation completely inhibits embryonic development. This inhibitory effect is partially alleviated by thymidine and more completely by thymidine plus hypoxanthine desoxyriboside(2). The inhibitory effect is also alleviated by high levels of "leucovorin," but not by folacin itself(3). These results were interpreted to mean that aminopterin acts in the chick embryo, as in other organisms, as an antagonist of folacin and its derivatives; that these substances are directly or indirectly essential for synthesis of thymidine and one or more purine derivatives; and that synthesis of these essential components of nucleic acid is the first process to become limiting in the aminopterin inhibited chick embryo. When thymidine, which is highly specific, and a suitable purine derivative are supplied preformed, these inhibited reactions are by-passed; nucleic acid synthesis, and hence initiation of embryonic development, can then occur in their absence.

The effectiveness of various purine bases and their nucleosides in enhancing the effects of thymidine is compared below. If the viewpoint cited above is correct, each of the effective purine derivatives must be able to serve as a precursor for synthesis of nucleic acid by the chick embryo.

Experimental. The procedures followed in

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TABLE I. Effect of Nucleic Acid Derivatives on Inhibitory Action of Aminopterin in the Chick Embryo.

Group No.	Compound injected, amt per egg*	No. of eggs	% embryos dying		% hatch
			0-5 days	6-20 days	
1	None	60	20	5	75
2	Water (.1 ml)	50	20	20	60
3	" (.2 ")	10	20	20	60
4	20 μg aminopterin	30	100	0	0
5	20 " " + 1 mg thymidine	30	83	7	10
6	As in 5 + .9 mg hypoxanthine	30	50	10	40
7	" + 2 inosine	20	25	15	60
8	" + 2 hypoxanthine desoxyriboside	20	60	20	20
9	" + .9 adenine	30	57	20	23
10	" + 2 adenosine	20	70	15	15
11	" + 1 adenine desoxyriboside	10	90	0	10
12	" + 1 guanine	30	57	17	27
13	" + 2 guanosine	20	80	15	5
14	" + 1 guanine desoxyriboside	10	100	0	0
15	" + 1 xanthine	10	80	10	10
16	" + .9 adenine and 1 mg guanine	30	37	37	27
17	" + .9 adenine and .9 hypoxanthine	30	30	33	37
18	" + 1 guanine and .9 hypoxanthine	30	33	23	43

* Thymidine (m.p. 185-86°), hypoxanthine desoxyriboside, adenine desoxyriboside, and guanine desoxyriboside were isolated from an enzymatic digest of desoxyribonucleic acid by slight modifications of published procedures(15,16). Other pure compounds were obtained from commercial sources.

injecting and incubating the eggs have been described(4). Injections were made prior to incubation in a total volume of 0.1 or 0.2 ml per egg. Where insolubility of compounds was a problem, they were dissolved and injected as sodium salts. Ten eggs were used per group in each experiment. At the level used, aminopterin frequently causes death prior to any blood formation. Upon gross examination such eggs cannot be distinguished from infertile eggs. For this reason, some infertile eggs may be included in the 0-5 day embryonic death classification.

Results. The combined results of 3 experiments are given in Table I. The non-injected control group average 75% hatch as compared with 60% for the water-injected control group, indicating a slight depressing effect of injecting water on hatchability. In confirmation of previous results(2), the injection of 20 μg of aminopterin prior to incubation resulted in 100% embryonic mortality prior to the fifth day of incubation, and this "toxic" effect is partially counteracted by simultaneous injection of 1 mg of thymidine. The activity of compounds such as thymidine is evidenced not only by the hatch of live chicks, but also in the definite increase in survival time of embryos which die before hatching.

Of the purine derivatives tested, inosine (hypoxanthine riboside) was easily the most effective in enhancing the effects of thymidine. The mixture completely counteracts aminopterin inhibition. Hypoxanthine desoxyriboside is active, but much less so than inosine. Lower concentrations of the desoxyribosides of adenine and guanine also were ineffective; the possibility remains that higher concentrations might be more effective.

Of the free purine bases, hypoxanthine is most active. Guanine and adenine each show distinct activity, however. Xanthine is inactive under the same conditions. Mixtures of the active purine bases show greater activity than the individual compounds; this enhanced activity may result in part, however, from the increased total concentration present.

Discussion. The participation of folic acid in the synthesis of purine bases and of thymine (or thymidine) was first suggested by the observation that certain lactic acid bacteria grew without folic acid if these nucleic acid derivatives were supplied(5-7). This same nutritional equivalence of nucleic acid derivatives and "leucovorin" can be demonstrated in the chick embryo, under the conditions used herein. The common feature in synthesis of the 2 groups of substances is now known to be incorporation of a one carbon unit (*e.g.* formate) in their molecules, for which a derivative of folic acid appears essential(7-9).

It is obvious that the small amounts of thymidine and purine bases injected in these experiments are not sufficient for synthesis of all of the nucleic acids of the hatched chick. From the figures of Novikoff and Potter(10) they should suffice, however, to permit development through the 8th day of incubation. It was shown earlier that the amount of aminopterin used here, 20 μg, is insufficient to prevent continued development if injected after the 8th day; the quantitative relationships are hence consistent with the interpretation made. If sufficient amounts of aminopterin were used to inhibit growth during the later phases of incubation, when rapid synthesis of comparatively large amounts of nucleic acids must occur, it is probable that reversal by the amounts of thymidine and inosine used here could not be accomplished.

If these interpretations are correct, the purine and pyrimidine bases that are effective in overcoming aminopterin inhibition must be directly utilizable as precursors of nucleic acids in the chick. The requirement here for thymidine, and the ineffectiveness of free thymine(2), correlates with the findings that bound pyrimidines, but not free pyrimidines are incorporated into the nucleic acids of rats (11,12). The superiority of hypoxanthine and especially inosine over other purine derivatives in potentiating the effects of thymidine correlates with the finding of Schulman *et al.*(13) that hypoxanthine appears in pigeon liver homogenates acting upon 4-amino-5-imidazolecarboxamide, and the postulate of Greenberg(14) that inosinic acid precedes hypoxanthine (which may not be an essential intermediate) in the biosynthetic pathway by which this system forms purine bases. In its preference for hypoxanthine and inosine, the chick embryo differs markedly from the rat, for which free hypoxanthine is not an effective precursor of nucleic acid(11). Adenine also appears readily utilizable by the chick embryo, as it is by the rat(11). In contrast to the rat, which does not utilize guanine for nucleic acid synthesis, the chick appears able to do so, and in this respect resembles the mouse(11). These interesting differences make it appear that the incubating egg would be an excellent additional tool for the elucidation of more intimate details of nucleic acid biogenesis through the use of isotopically marked intermediates.

Summary. Several purine bases and related nucleosides were tested for their effectiveness in potentiating the reversal by thymidine of aminopterin inhibition of the chick embryo. Of the free purine bases, hypoxanthine was most active; adenine and guanine were also active, while xanthine was inactive. Of the nucleosides tested, only inosine was active. Inosine was more active than hypoxanthine; together with thymidine it completely overcame the effects of the low level of aminopterin employed. The results are interpreted to mean that aminopterin prevents embryonic growth by preventing synthesis of compounds essential for formation of nucleic acids; by supplying these substances preformed, growth occurs even though aminopterin is present. It follows that thymidine, and the active purine bases and derivatives listed above, are utilizable for nucleic acid synthesis by the chick embryo.

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Inhibition of Pancreatic Secretion by the Carbonic Anhydrase Inhibitor, 2-Acetylamino-1,3,4-Thiadiazole-5-Sulfonamide (Compound No. 6063).* (19767)

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Carbonic anhydrase, the enzyme concerned with the hydration of CO_2 and, consequently, the formation of bicarbonate, occurs in significant concentration in the pancreas(1). Tucker and Ball(2) attempted to demonstrate that carbonic anhydrase plays a major role in the formation of bicarbonate by the pancreas. For this purpose, sulfanilamide, an inhibitor of the enzyme *in vitro,* was injected into anesthetized dogs with cannulated pancreatic ducts secreting in response to secretin. Failure of these efforts led the investigators to conclude that this enzyme plays no essential role in pancreatic secretion. Recently, a new carbonic anhydrase inhibitor (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide, designated compound No. 6063) which may be as much as 440 times more potent than sulfanilamide *in vitro,* has become available(3,4). This agent has already been found to reduce the secretion of histamine-induced gastric HCl considerably, when administered intravenously to Heidenhain pouch dogs(5), or when applied to the mucosa topically(6). Hence, we undertook to study the effect of this drug on pancreatic secretion, and the results are reported herewith.

Methods and materials. Observations were made on 3 mongrel dogs, provided with duodenal fistulae for collection of pancreatic juice according to Thomas(7). Experiments were never done less than 4 weeks after operation, nor at intervals of less than one week. The dogs were fasted 24 hours prior to each experiment. Following cannulation of the main pancreatic duct, unstimulated secretion was collected for one hour. Then Secretin, Lilly (5 units/kg) was injected intravenously and the secretory response was followed for one hour (control period, A). Immediately after this second hour, the inhibitor, dissolved in a minimum of NaOH solution, was injected slowly intravenously. Two or 3 hours after the inhibitor had been administered, a second injection of secretin was given and the secretion collected for the following hour (period B). The same procedure was repeated 5 hours after injection of the inhibitor (period C). Pancreatic juice specimens were collected for successive 10-minute intervals and all volumes were recorded; those containing less than one ml were pooled consecutively until a specimen with at least this volume was obtained. The concentration of total CO_2 (*i.e.,* dissolved gas plus bicarbonate) in each sample was determined in duplicate, using the constant volume method of Van Slyke(8). All determinations were performed with as little delay as possible —never more than 5 hours after collection. In all, 18 experiments were performed, using compound No. 6063 in dosages of 2, 5, 10, 30, 40 or 60 mg/kg. There were no general toxic signs following the slow intravenous injection of the drug, in conformity with previous observations(9) in this laboratory.

The extent of inhibition was measured in terms of volume of secretion and bicarbonate concentration, by comparison of the *hourly* response to secretin for period A (without inhibitor) with the responses for periods B and C. Percentage inhibition for each experiment was calculated in terms of the decrease

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in hourly response relative to the corresponding control period.

Results. There was clear evidence of a reduction in volume response to secretin following the intravenous administration of compound No. 6063 in all experiments, except one at the lowest dosage level. Of the remaining 17 experiments, the degree of inhibition was less than 20% in 1, 20-50% in 7, 51-70% in 3, and 71-96% in 6. It is noteworthy that 13 of these 17 experiments show a greater degree of inhibition in period B than in period C.

Bicarbonate concentration was also reduced in most of the experiments, the percentage inhibition being distributed as follows: 0-10% in 2 experiments, 11-20% in 5, 21-40% in 7, and 41-60% in 4 experiments, but none over 60%. In period A, with secretin alone, the concentration of bicarbonate varied directly with the volume-rate of secretion. in conformity with previously published studies. In periods B and C, following administration of the carbonic anhydrase inhibitor, the bicarbonate concentration was generally lower than would be expected from the relation in period A. Hence, it appears that the reduction in bicarbonate concentration effected by the inhibitor cannot be attributed solely to the corresponding decrease in volume, but is greater than would be expected solely from the control concentration-volume correlation.

For both volume and bicarbonate concentration, the magnitude of the inhibition appeared to be unrelated to the dosage of compound No. 6063 in the range studied.

Comment. The results of this study demonstrate the influence of a drug, which is a potent carbonic anhydrase inhibitor *in vitro*, on pancreatic secretion *in vivo*. It is conceivable that the reduction in secretion resulted only indirectly, that is, from the influence of compound No. 6063 on some other bodily function which in turn affected pancreatic activity, but our experience with this inhibitor in gastric secretion suggests that this is unlikely. The highest degree of volume inhibition attained was 95%; it never reached 100%. The residual 5% may possibly be explained in part as representing the secretion of bicarbonate formed from the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ in the absence of catalysis. Another important consideration is the concentration of the drug within the pancreatic cell, which may not have sufficed to inhibit its entire content of carbonic anhydrase.

Summary. Intravenous injection of 2-acetylamino - 1,3,4 - thiadiazole-5-sulfonamide (compound No. 6063) in pancreatic fistula dogs results in a marked reduction in the volume output and the bicarbonate concentration of pancreatic secretion following stimulation with secretin. Since this compound is a potent inhibitor of carbonic anhydrase activity *in vivo,* it is likely that this reduction in secretory activity results from a similar inhibition of the carbonic anhydrase present in the pancreas. This supports the view that this enzyme plays a dominant role in bicarbonate formation by this gland.

Statistical analyses of the concentration-volume data will be published later in a detailed report of this work.

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Effect of Hypophysectomy on Size of Remaining Kidney after Ureteroduodenostomy and Contralateral Nephrectomy. (19768)

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This study was prompted by an examination of the adrenal glands of a number of dogs and rats that Drs. M. A. Block, K. G. Wakim and F. C. Mann had used in another investigation. They had removed one kidney and had transplanted the ureter of the remaining kidney into the duodenum of each animal. In every animal, the adrenal glands and the remaining kidney were appreciably heavier than those of control animals. This observation inspired an inquiry into the possible factors responsible for the increased size of these organs. Among the various possibilities, the influence of the hypophysis cerebri was considered to be the most probable. Consequently, experiments were designed to test the validity of this idea.

Method of study. Seven adult dogs, both males and females, weighing from 8 to 14 kg, were used. The pituitary body was approached by the transbuccal procedure. The operation was done with the animals anesthetized with pentobarbital sodium and with the usual sterile technic. In 4 of the 7 dogs, the pituitary body was successfully removed, while in the remaining 3 it was not removed because of difficulties with hemorrhage at the time of operation. In these 3 dogs, the pituitary body was found intact at necropsy. These dogs served as control animals.

The most reliable criterion of hypophysectomy is the absence of the pituitary body upon macroscopic and microscopic examination. In the 4 cases in which hypophysectomy was successful, no traces of the pituitary body were found grossly or upon microscopic examination of sections of the tissue removed from the site of the organ. In animals living more than 21 to 28 days after hypophysectomy, additional evidence of the absence of the pituitary body was shown by the size and histologic appearance of such structures as the adrenal cortex, thyroid gland and gonads.

In from 12 to 43 days after hypophysectomy, the left kidney was removed from each of the 7 animals and served as a control for the study of the remaining kidney. The operation was done with ether anesthesia, and the usual sterile precautions were followed. The kidney was removed through a midline incision of the abdominal wall. Immediately after its removal, the kidney was incised on its outer border and was placed in a 10% solution of formalin, and was weighed 24 hours later, after removal of all excess tissues.

The ureteral transplantation, which was done according to the method described by Beaver and Mann(1), was performed immediately after the left kidney had been removed. In cases in which hypophysectomy was performed, the kidney was not removed until 5 to 40 days had passed.

Histologic preparations were made of all the kidneys, adrenal glands, thyroid glands and gonads, and serial sections were made of all tissue found at postmortem on the floor of the sella turcica and in the region of the attachment of the hypophysis cerebri.

Dogs 1, 2 and 4 of the hypophysectomized group and dog 3 of the nonhypophysectomized group (Table I) were found dead, but, since their deaths were discovered before postmortem changes occurred, their kidneys and other organs were given the same treatment as those of the other animals in the series.

The percentage increase in the weight of the remaining kidney was calculated by use of the following formula:

$$\%\ \text{enlargement of remaining kidney} = \frac{K_2w - K_1w \times 100}{K_1w}$$

in which K_2w equals the weight of the remaining kidney and K_1w equals the weight of the control kidney.

Results. Evaluated according to the formula, the remaining kidneys of the control dogs or the dogs in which the hypophysis

TABLE I. Effect of Hypophysectomy on Size of Remaining Kidney after Transplantation of Its Ureter into Duodenum and Removal of Contralateral Kidney.

Hypophysectomized dogs

Dog	Sex	Body wt, kg: Before hypophysectomy	Body wt, kg: Before abdominal operation	Body wt, kg: Final	Wt of kidneys: Left, g	Wt of kidneys: Right, g	Wt of kidneys: Difference, g	Wt of kidneys: Difference, %	Period of survival, days: After abdominal operation	Period of survival, days: After hypophysectomy
1	♂	9.9	9.5	9.5	32	39	7	21.8	7	12
2	♂	10.5	10.25	9.25	33	39	6	18.2	4	10
3	♂	14.2	12.4	11.2	27	40	13	48.1	3	43
4	♀	12.25	13	13	24	27	3	12.5	8	37
Mean		11.7	11.2	10.7	29	36.25	7.25	25.15	5.5	25.5

Nonhypophysectomized dogs

Dog	Sex	Body wt, kg: Initial	Body wt, kg: Before abdominal operation	Body wt, kg: Final	Wt of kidneys: Left, g	Wt of kidneys: Right, g	Wt of kidneys: Difference, g	Wt of kidneys: Difference, %	Period of survival, days: After abdominal operation	Period of survival, days: After transbuccal operation
1	♂	11.5	11.5	10.25	25	57	32	128	14	37
2	♂	8.4	8.4	7.5	20	44	24	120	7	13
3	♀	9.5	9.5	9.7	22	37.5	15.5	70	6	32
Mean		9.8	9.8	9.15	22.3	46.1	23.8	106	9	27.3

cerebri was intact were much larger and heavier than those of the hypophysectomized dogs. The increase in weight of the kidneys of the control nonhypophysectomized dogs ranged from 70 to 128% as compared to 12.5 to 48.1% for the hypophysectomized animals. The mean percentage difference was 106 in the control group as compared to 25.15% in the hypophysectomized dogs (Table I). This difference is highly significant statistically (T = 4.9, with 5 D.F.: $P < 0.01$).

The control kidneys of the hypophysectomized dogs were smaller than those of the nonhypophysectomized dogs. The remaining kidneys were the same in appearance. They had a dilated ureter and pelvis and accentuated renal capsular vessels. The kidneys of the hypophysectomized dogs were affected to a lesser degree than those of the nonhypophysectomized dogs (Fig. 1).

The control, or the left kidney, of the hypophysectomized dogs showed diminished capsular space, and many of the renal tubules seemed to have narrow lumina.

The remaining kidneys of the nonhypophysectomized dogs showed degeneration of the renal tubular cells with marked dilatation of their lumina. In the remaining kidneys of the hypophysectomized dogs, the same changes were seen, but to a lesser degree.

The adrenal glands (Fig. 2), gonads and the thyroid glands appeared smaller, and showed changes consistent with the ablation of the pituitary body in dogs living more than 3 or 4 weeks after hypophysectomy.

Comment. The results of the experiments on nonhypophysectomized dogs in which one kidney was removed and the ureter of the other was transplanted into the duodenum are very similar to those reported by Bollman and Mann(2), and by Block, Wakim and Mann(3). The former authors reported a 60 to 100% increase in weight of the kidney within 9 days, and the latter authors reported a 63.5 to 190.0% increase within 72 to 220 hours. In this study, the increase in the weight of the remaining kidney ranged from 70 to 128% in the nonhypophysectomized dogs in 6 to 14 days as compared to 12.5 to 48.1% in hypophysectomized dogs within 3 to 8 days.

In considering these striking results in the hypophysectomized group of animals, an inquiry can be made as to the possible relation of changes in body weight of the animals to the changes in the weight of the kidneys. Since there was an increase in the body weight of 1 dog and a decrease in the body weight of another, and since there was little change in the other 2 dogs, there is not much basis for further pursuit of this point.

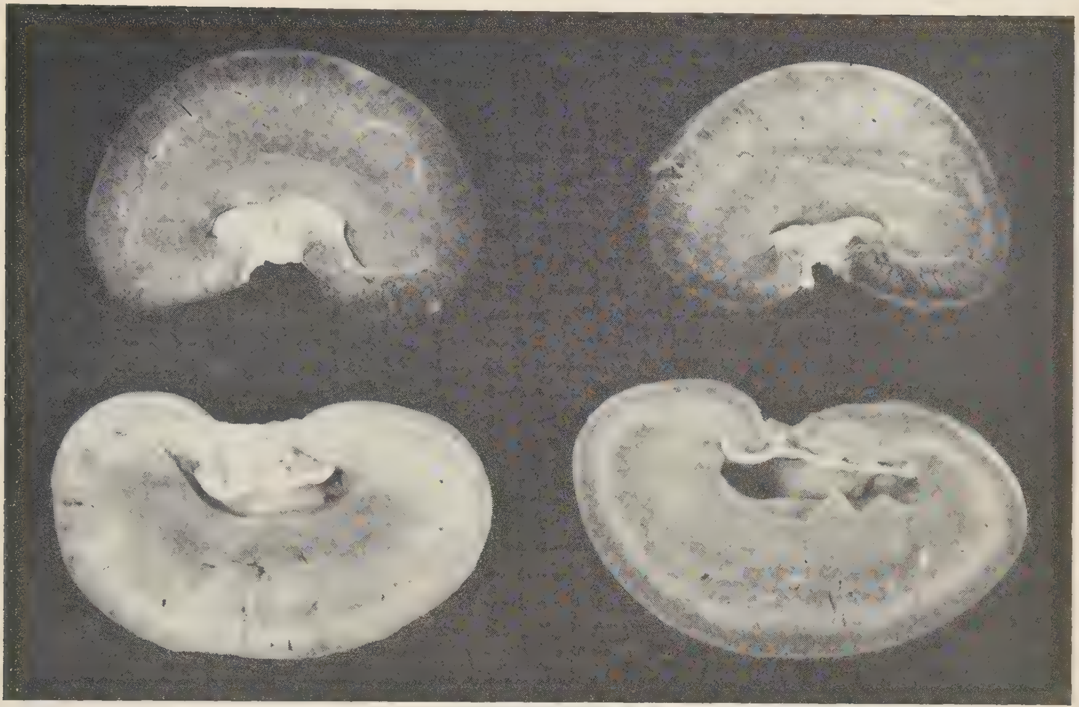

FIG. 1. Control kidney (above) and the remaining kidney (below) of hypophysectomized dog (left) and nonhypophysectomized dog (right). One may compare the dilatation of the renal pelves of the two kidneys.

If the position is taken that an explanation of the results resides in the lack of the hypophysis cerebri, and if this position is accepted, it may legitimately be asked whether it is a specific and direct effect due to a lack of a renotropic principle or whether the effect is indirectly mediated through one or another or through a combination of many of the endocrine glands or is due to the lack of the renotropic effect of the pituitary "tropes." That there may be a direct pituitary influence (renotropic) on the kidney has been suspected [Selye(4)] but never demonstrated. The results of the present study seem to point to such a mechanism.

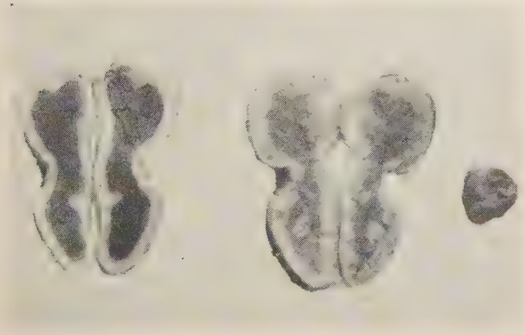

FIG. 2. Adrenal glands of hypophysectomized dog (left) and intact animal (right) with the pituitary body removed at necropsy. One may compare the cortices of the 2 adrenal glands. (Thirty-seven days after hypophysectomy.)

In the absence of the pituitary body, the size of the kidney was found to be smaller than the control in dogs [Brown(5); Dandy and Reichert(6)] and in rats [Smith(7); McQueen-Williams and Thompson(8); Selye (4) and Astarabadi and Essex(9)]. Opinions in regard to the occurrence of compensatory hypertrophy in the absence of the pituitary body are controversial, but the consensus presented by Houssay(10) is that the pituitary body is considered important but not essential for the compensatory renal hypertrophy and, perhaps, it is the summation effect of all the pituitary hormones which plays the role and not a special renotropic hormone. However, the factors contributing to the increase in the size of the remaining kidney following transplantation of its ureter into the duodenum with contralateral nephrectomy has been discussed by Wakim, Block and Mann (11), and the evidence might be interpreted to indicate that the kidney was not carrying on efficient physiologic functions.

Under the condition of hypophysectomy in animals, and hypopituitarism in man, disturbance in renal physiology has been reported. For instance, the kidney showed marked depression of water diuresis in man [Slessor(12) and Robertson(13)], and similar findings in dogs have been reported by White(14); Lane and de Bodo(15); Earle, de Bodo, Schwartz, Farber, Kurtz and Greenberg(16); de Bodo, Schwartz, Greenberg, Kurtz, Earle and Farber(17), although White found that the somatotropic hormone restored the depressed function of the kidney of the hypophysectomized dog to normal and even caused hyperactivity.

From the evidence available, it would seem that the hypophysis cerebri must influence both the size and function of the kidney but through what mechanism is not clearly and completely understood.

Summary. Hypophysectomy was performed on adult male and female dogs by using the buccal approach. The left kidney which was removed through a midline abdominal incision served as a control, and the ureter of the right kidney was transplanted to the duodenum. The remaining kidney was removed 3 to 14 days later, and its weight and cytologic structure were compared with those of the control kidney.

The remaining kidneys of the nonhypophysectomized dogs weighed an average of 106% more than the control kidneys. This is in contrast to a 25.15% average increase in the weight of the remaining kidneys over the control kidneys of the hypophysectomized dogs.

Our thanks are due to Dr. Peter B. Dews for his help in analyzing the data statistically. We also wish to thank Mr. W. A. Meeker and Mr. Walter Ogg for their technical assistance.

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Antimicrobial Activity of a Natural and a Synthetic Polypeptide.* (19769)

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In an attempt to isolate factors concerned in the natural resistance of experimental animals to infection with *Bacillus anthracis*, Bloom, *et al.*(1) isolated an antibacterial basic protein characterized by a high content of lysine. Active fractions were isolated from polymorphonuclear leucocytes, caecum, thymus, and the pancreas of various species of animals. Later, Bloom and Blake(2) found the active fraction in thyroid and more recently it has been isolated from spleen(3). In addition to *Bacillus anthracis*, these fractions are active against *Escherichia coli, Staphylococcus aureus, beta*-hemolytic streptococci and *Bacillus megatherium*(2,3). Impressed with the high lysine content of these tissue polypeptides, Weissman and Graf(4) compared the antibacterial activity of a histone containing 16.4% lysine with a tissue polypeptide which had a lysine content of 30%. Expressing their results in terms of milliequivalents of lysine per unit of antibacterial activity, they found a correlation between the content of lysine and the antibacterial activity. These results led Stahmann *et al.*(5) to synthesize lysine polypeptides for the purpose of studying their antimicrobial activity. As predicted, these synthetic lysine poylpeptides had high antimicrobial activity when tested *in vitro*(5,6).

The purpose of this communication is to present evidence which indicates that the antimicrobial activity of the tissue polypeptide resides in lysine residues within the molecule. These results were obtained by comparing the antibacterial and antiviral activity of the natural tissue peptide with that of a synthetic lysine polypeptide. Further evidence was obtained by studying their neutralization and complex formation with desoxyribonucleic acid (DRNA) and ribonucleic acid (RNA).

Methods. The antimicrobial activity of these substances was tested with a system comprising bacteriophage (bacterial virus) type A and its host *Clostridium madisonii* McCoy, strain 16. The assay method, including the composition of the medium, the preparation of the stock virus and methods for cultivating the host are given in detail elsewhere(7). The tissue polypeptide was prepared by the method of Bloom, *et al.*(1) from calf thymus, while the synthetic lysine polypeptide† was prepared by a modification of the method of Katchalski, *et al.*(8). Although both the hydroiodides and the hydrochloride salts were used, all of the work reported here was carried out with the hydrochloride. The concentrations were based on the weight of the free base. This particular sample of synthetic polypeptide contained, on the average, 85 lysine residues. The DRNA was prepared by the method of Mirsky and Pollister(9) with the protein extraction of the nucleoprotein as reported by McCarty(10). The DRNA contained 13.4% nitrogen and 8.0% phosphorus; the RNA contained 13.6% nitrogen and 8.5% phosphorus. These materials were dissolved in distilled water and the pH adjusted to 7.0 with a dilute solution of NaOH. Since *Clostridium madisonii* was employed in the assay of virus, it was necessary to determine the influence of two peptides on this particular host. In previous studies (1,2), the antibacterial activity was determined by measuring inhibition of aerobic respiration. In the present investigation, the inhibition of cellular multiplication was de-

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* A portion of this work was completed in the Department of Bacteriology, University of Wisconsin and supported by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. Supported also in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† We wish to thank Dr. M. A. Stahmann of the Department of Biochemistry, University of Wisconsin for the synthetic lysine polypeptide.

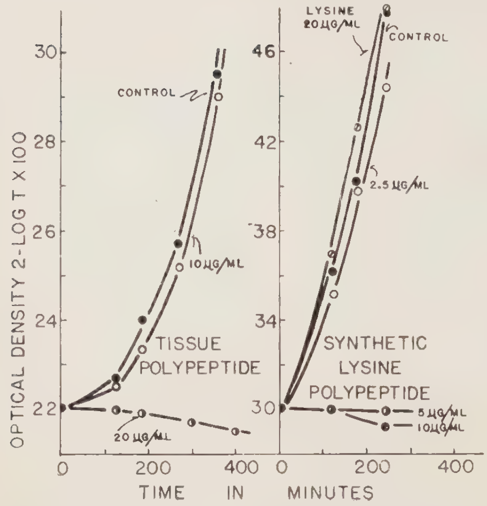

FIG. 1. Determination of relative antibacterial activity of tissue and synthetic polypeptides and lysine employing *Clostridium madisonii.*

termined as an index to the amount of antibacterial activity of the peptides. This was accomplished by the determination of optical density using a Coleman "junior spectrophotometer" in a manner previously described(7).

Experimental. In Fig. 1, the antibacterial activity of the tissue polypeptide and synthetic lysine polypeptide are compared, employing the bacteriophage susceptible host, *Clostridium madisonii.* The conditions and methods used in this experiment relative to the composition of medium and preparation of inocula were similar to those used in the viral assay (7). The initial concentration of bacterial cells was in the order of 1.0 to 1.5 x 10^8 organisms per ml; these were added to several tubes each containing 18 ml of sucrose medium adjusted to pH 6.5 and maintained at a temperature of 34°C. To each of these tubes was added the indicated concentrations of tissue polypeptide and synthetic lysine polypeptide. The degree of inactivation was followed by frequent determinations of the changes in optical density. It can be seen that the quantity of tissue polypeptide required to inactivate completely this concentration of cells falls somewhere between 10 and 20 micrograms per ml. The amount of synthetic lysine polypeptide, however, required to inactivate the same quantity of cells was between 2.5 and 5 μg per ml. From these results, one can conclude that the synthetic lysine polypeptide has approximately 4 times the activity of the natural tissue polypeptide.

Antiviral activity of tissue and synthetic lysine polypeptide. The comparative antiviral activity of these preparations was determined in a solution consisting of 3 g of $(NH_4)_2HPO_4$ and 6 g of KH_2PO_4 per liter of distilled water adjusted to pH 6.5. Eight ml of this solution was placed in each of a series of tubes. To each of these tubes was added 1 ml containing 1 x 10^8 units of virus; these tubes were then divided into 3 groups. As indicated in Fig. 2, tissue polypeptide, synthetic lysine polypeptide and lysine were added at the indicated concentrations to each of the tubes within each group. The tubes were then incubated at 34°C for 30 minutes. At the end of this period, a portion of the mixture was assayed for active virus. It may be seen from Fig. 2 that the synthetic lysine polypeptide was highly antiviral, 10 μg inactivating in excess of 99% of the added virus. If one examines the slopes of the inactivation curves, it is immediately apparent that the synthetic lysine polypeptide is approximately 4 to 5 times more active than the tissue polypeptide on a weight basis. Lysine, run as a control, at concentrations in excess of 30 μg per ml did not inactivate this bacterial virus.

The neutralization of synthetic lysine polypeptide and tissue polypeptide by DRNA and

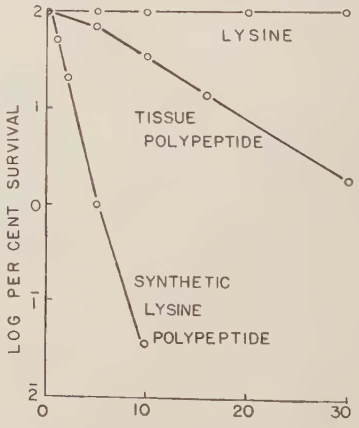

FIG. 2. Determination of the relative antiviral (bacteriophage) activity of tissue and synthetic lysine polypeptides and lysine. Initial concentration of virus, 1 × 10^8 units per ml. Exposed for 30 min at 34°C.

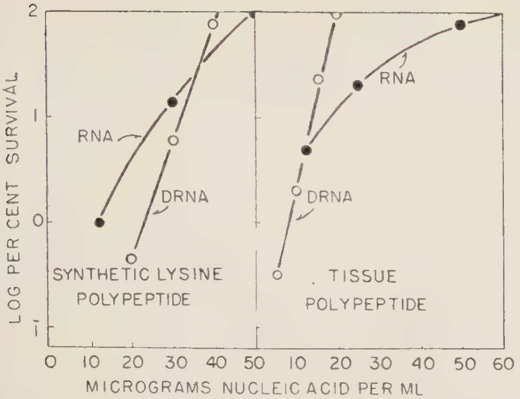

FIG. 3. Neutralization of synthetic lysine polypeptide (10 μg per ml) and tissue polypeptide (30 μg per ml) by DRNA and RNA as determined by the inhibition of antiviral effect.

RNA. Previous investigators have reported that DRNA combines with the tissue polypeptide stoichiometrically, while RNA combines in multiple proportions, depending upon the relative concentrations of the reactants (11). It was of interest, therefore, to determine the ability of RNA and DRNA to neutralize the synthetic lysine polypeptide and to compare the results in a similar experiment with tissue polypeptide. The experiment comprised 4 series of tubes divided into two groups. Into each tube of the first group was placed 8 ml of medium containing 10 μg per ml of synthetic lysine polypeptide. Likewise, 30 μg per ml of tissue polypeptide was placed into each tube of the second group. RNA, at the concentrations indicated in Fig. 3, was placed in the first series of Groups 1 and 2, while the given concentrations of DRNA were placed in the second series of Groups 1 and 2. The contents of the tubes were mixed thoroughly and bacterial virus was added to each tube to give a final concentration of 1 x 10^8 units per ml. The tubes were then incubated at 34°C for 30 minutes. At the end of this period, aliquots were removed from each tube and assayed for the amount of active virus remaining. The results given in Fig. 3 are plotted as log per cent survival of virus against the number of micrograms of nucleic acid, either DRNA or RNA, added to each tube. The amount of free virus remaining is a sensitive indicator of the concentration of uncombined polypeptide as previously shown in Fig. 2. Results given in Fig. 3 show that 40 μg of nucleic acid (DRNA) neutralize completely 10 μg of synthetic lysine polypeptide. On the other hand, about 18 μg of nucleic acid (DRNA) neutralize 30 μg of tissue polypeptide. The linearity of these curves indicates that the DRNA is combining with both the tissue polypeptide and synthetic lysine polypeptide stoichiometrically. The nonlinearity of the RNA curves might indicate that this nucleic acid does not combine with either the tissue polypeptide or the synthetic lysine polypeptide stoichiometrically, but rather in multiple proportions depending upon the relative concentrations of the two reactants. As noted previously (11) and in these experiments, when increasing concentrations of RNA is added to constant amounts of synthetic lysine polypeptide, a precipitate forms which increases to a maximum and then sharply declines as the ribonucleic acid concentration is increased. The results of such an experiment are given in Fig. 4 where the precipitate is recorded as optical density and plotted against the number of μg of RNA added to each of a series of tubes, each containing 10 μg of the synthetic lysine polypeptide. As the RNA is increased beyond 25 μg per ml the complex formed between RNA and the peptide either dissociates or forms soluble complexes. To test these two possibilities, aliquots were removed from each tube after a suitable incubation period and tested for free virus. The amount of virus remaining is a test for the amount of free peptide. It can be seen in Fig. 4 that as the RNA increases, the amount of free peptide decreases, until at approximately 50 μg of RNA, 100% of the virus is recovered, indicating that nearly all of the peptide has been neutralized. It can also be seen that at concentrations of RNA where there is a minimum of precipitate formed, all of the peptide must be incorporated in soluble complexes because nearly 100% of the virus is recovered in these tubes. This would indicate, therefore that in the presence of excess RNA a soluble complex is formed which results in the complete neutralization of the synthetic lysine polypeptide.

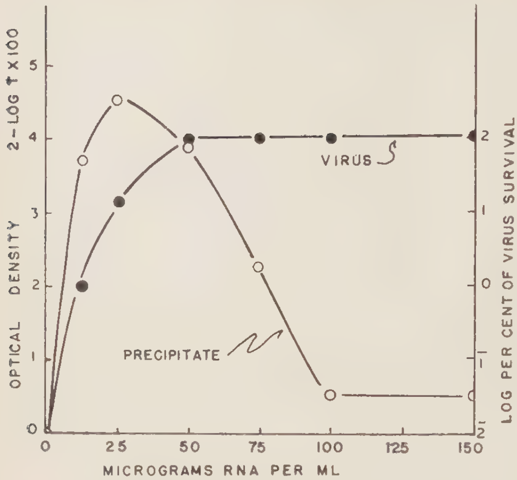

FIG. 4. Quantitative titration of synthetic lysine polypeptide (10 μg per ml) with RNA and the demonstration of insoluble and soluble complexes as determined by optical density and the inhibition of antiviral effect.

Discussion. These comparative studies with synthetic lysine polypeptide and tissue polypeptide lend significance to the high content of lysine in the natural tissue polypeptide. The fact that the synthetic lysine polypeptide is approximately 4 to 5 times more active than the tissue polypeptide when compared on a weight basis, but of comparable activity when compared on the number of meq. of lysine within the molecule suggests that the activity of the natural peptide resides in the lysine portion of the molecule. The comparable types of interaction between DRNA and RNA with the two polypeptides lends further support to this implication of lysine as the active portion of the natural peptide. These findings are not in conflict with the previous postulation on the possible mechanism of action(12). These polypeptides have high isoelectric points and are, therefore, positively charged at pH 6.5; on the other hand, substances such as DRNA and RNA are negatively charged. It can be assumed, therefore, that the phosphoral groups within DRNA combine stoichiometrically with the epsilon amino group of the lysine residues within these peptides. The interaction between RNA and these peptides seems somewhat more complicated and more difficult to explain. This latter interaction seems to be quite comparable to an antigen-antibody reaction. The curve in Fig. 4 representing the amount of precipitate formed when varying amounts of ribonucleic acid are added to constant amounts of synthetic lysine polypeptide is quite comparable to that obtained when antibody concentration is maintained constant, increasing the quantity of antigen added. In both cases it appears that soluble complexes are formed. The similarity goes further, in that Lewis, *et al.*(11) have shown that if one determines the nitrogen-phosphorus ratio of the resulting precipitate at varying places along a similar curve, one finds that the ratio varies depending upon the relative concentration of the reactants. A comprehensive study of this phase of the investigation comparable to that reported by Bjornesjo and Teorell(13) for the DRNA-oval-albumin and DRNA-histone systems is anticipated.

The efficacy of the tissue and the synthetic polypeptides when tested for their antimicrobial activity *in vivo* has not been promising (1,6). This is not surprising when one considers their high affinity for such substances as RNA, DRNA and other negatively charged high molecular weight substances commonly found in tissues. This fact, however, does not preclude the importance of the tissue peptide in the natural resistance of the host to microbial infections as originally postulated (14). It is possible that as a result of the host-parasite interaction and the resulting tissue damage, the peptide is liberated, destroying the parasite within the immediate environment of the necrotic tissue.

If one assumes that this tissue peptide is liberated as a result of tissue necrosis, it could also function in the formation of fibrinoid in a manner postulated by Altshuler and Angevine(15). This tissue polypeptide combines with the acidic polysaccharides, hyaluronic acid and heparin, to form insoluble complexes. Whether these insoluble complexes have the chemical and physical properties of fibrinoid has not been determined.

Summary. The antibacterial and antiviral properties of a tissue polypeptide were compared with the activity of a synthetic lysine polypeptide. On a weight basis, the activity of the synthetic peptide was approximately

4 times greater than that of the natural peptide. From the fact that the tissue peptide contains 30% lysine, it is possible to conclude that the activity resides within the lysine portion of the molecule. Additional evidence to support the implication of lysine was obtained from a comparative study on the mode of inactivation of these peptides by desoxyribonucleic acid (DRNA) and ribonucleic acid (RNA). Both peptides combine stoichiometrically, at pH 6.5 with DRNA but not with RNA; it is apparent that both peptides combine with RNA in multiple proportions depending upon the relative concentrations of the reactants. In this respect, the similarity between the RNA-peptide interaction and antigen-antibody reactions is pointed out. A mechanism by which the tissue polypeptide could take part in the natural resistance of animals to infection and the possible role of these basic peptides in fibrinoid degeneration is discussed.

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Use of Radioactive Iodinated Human Serum Albumin as Tracer Agent in Study of Cerebral Disorders.* (19770)

THOMAS W. FARMER, H. LANZ, MARY S. MCCALL, CAROL MORGAN, W. NICHOLL, AND R. CLAYTON. (Introduced by N. A. Womack.)

From the Department of Medicine, S. W. Medical School of the University of Texas, and the Radioisotope Unit, Veterans Administration Hospital, Dallas, Texas.

During the past 4 years diiodofluorescein (DIF)(1-7), radioactive potassium(8,9), iodinated human serum albumin (RIHSA)(10), sodium iodide(11), and radioactive copper (12) have been used in the study of intracranial lesions by the isotope-encephalometric technic. An increased radioactivity at a particular site has been used as evidence of the possible presence of a neoplasm. The reported accuracy of localization of tumors has varied from 5%(13) to 95%(5). Technics have varied with advances in the instruments used. Emphasis has been placed on the study of patients with known or suspected tumors. In previous studies with DIF by the authors (14) increases in uptake above 10% were observed in 21 of 25 cases of cerebral neoplasm, diagnosed by operation or autopsy. However, wide variations in uptake on repeated studies were observed in some of the patients with neoplasms. Accurate studies of control groups were difficult due to the rapid excretion of

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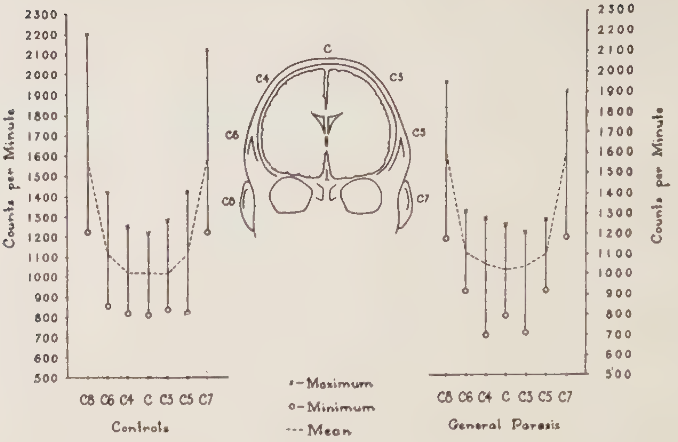
* This investigation was supported in part by a research grant from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.



Variations in Uptake at "C" Positions From Patient to Patient

FIG. 1.

DIF. For this reason the present studies were undertaken with RIHSA. Chou *et al.* (10) have emphasized that one of the advantages of RIHSA is that it remains in the tissues for several days. This permits repeated counting on each patient.

It is the purpose of this communication to present an analysis of data obtained with reference to: 1) variations in the level of uptake of RIHSA in normal individuals and in patients with diffuse cerebral diseases, and 2) variations in uptake from symmetrical paired positions in normal individuals, patients with diffuse cerebral disease, and patients with focal cerebral neoplasms.

Method. All patients were given 10 drops of Lugol's solution 3 times daily for 2 days prior to the intravenous injection of a standard dose of 200 microcuries of RIHSA.[†] A survey of the radiation arising from within the skull 24 hours after injection was made by placing a shielded GM tube over 32 standard areas (6 midline and 13 symmetrical, paired positions). One minute counts were taken at each position. An elastic rubber bathing cap was placed over the head, and all points were measured to be certain of accurate symmetrical positioning. A Mark 1, Model 13, bismuth cathode end-window Geiger-Muller tube was used (Radiation Counter Laboratories). It was held in a cylindrical lead shield of one-half inch thickness. This tube assembly was placed on the area to be measured in such a way that the tube axis was normal to the skull. The pulses from the tube were fed directly into a scaling circuit, and readings were obtained in counts per minute. When significant differences in uptake between symmetrical paired positions were observed these counts were checked until reproducible results were obtained.

Results. 1. *Variation in the level of uptake of RIHSA at any given point.* In 10 normal healthy adults and 13 patients institutionalized with general paresis the uptakes in each individual at 32 sites were studied following the injection of RIHSA. The uptakes obtained in the seven positions extending from the left temporal to the right temporal area are plotted in Fig. 1. In the group of controls a wide variation from person to person in the absolute counts per minute with constant dosage was observed. For example, in the C8 position the counts varied from 1200 to 2200 counts per minute. The dotted line represents the arithmetic mean at each of the 7 positions. This describes a symmetrically concave curve and is similar to the pattern observed in individual uptakes. In the group of patients with general paresis (Fig. 1) the variation in levels of uptake is similar to that observed in the normal group. Also the curve

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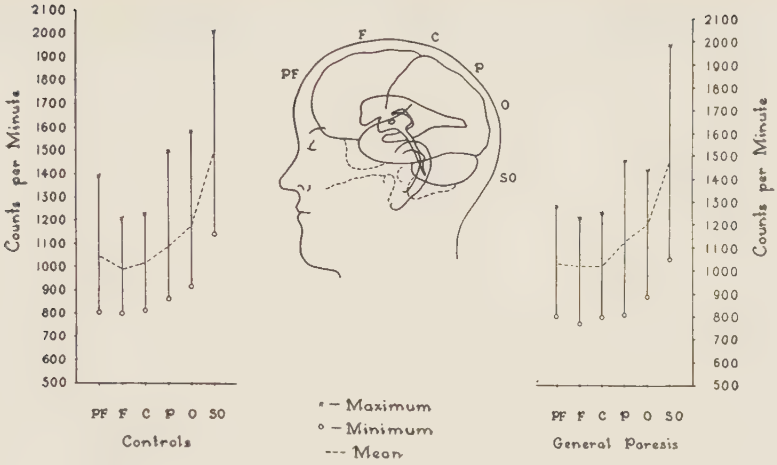
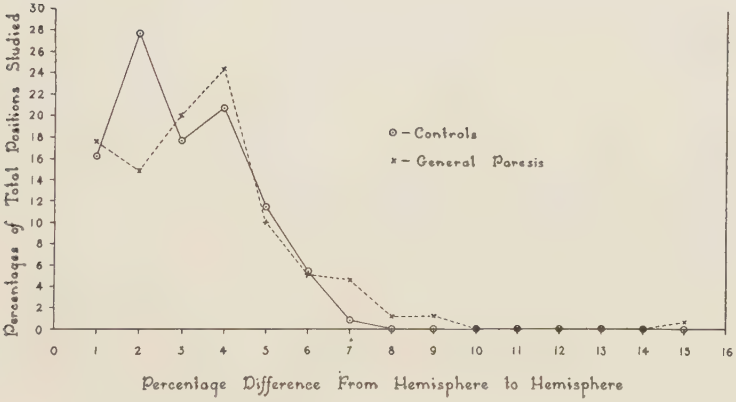
[†] Obtained from the Abbott Laboratories, North Chicago, Ill.



Variations in Midline Uptake From Patient to Patient

FIG. 2.



Variations in Percentage Uptake Within the Same Patient

FIG. 3.

of the means for this group is almost identical with the control group.

The level and range of uptake of the 6 median sagittal positions are presented in Fig. 2. In the normal controls there is a wide range of variation in uptake. The mean at each point is plotted along a dotted concave curve. This curve is similar to the pattern observed in individual uptakes. In the patients with general paresis the variation in level of uptake was similar to that observed in the normal group. The curve of the means is almost identical with that in the normal group. This variation was not altered when correction was made for the patients' weights.

2. *Variation in the uptake of RIHSA at symmetrical paired positions in the same individual.* The percentage differences in uptake between the 13 paired symmetrical positions in each of 10 controls and 13 patients with general paresis were determined. The frequencies with which increasing percentage differences were obtained are presented in Fig. 3. In the control group the greatest difference observed between paired positions was 7%. In the group with general paresis a similar distribution was observed with an

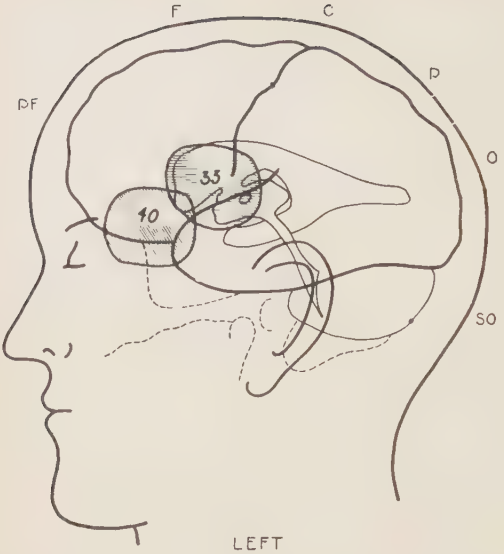


FIG. 4. Localization of meningioma using RIHSA. Figures in shaded areas indicate % increase in radioactivity over symmetrical position on the right side.

occasional difference as high as 9%, and one of 15%.

In patients with cerebral neoplasms which show an increased uptake of RIHSA the percentage differences in symmetrical positions varied widely—from 12 to 64%. A representative example is presented in Fig. 4.

Discussion. The possible localization of cerebral neoplasms by various methods using radioactive materials has been studied by many investigators in the past four years. If a sufficiently accurate method were available it would have a wide range of clinical usefulness. The present studies indicate that with the methods used there is a wide variation in uptake of RIHSA by the brain. This is related both to biological factors, such as the distribution of RIHSA in the body tissues and to technical factors. The significance of this method in the localization of cerebral neoplasms depends upon more extensive studies of normal individuals and patients with a wide variety of cerebral disorders in addition to patients with cerebral neoplasms. These studies are in progress.

Summary. 1. The use of RIHSA in studies of cerebral disorders is presented. With a 200 μc dose of RIHSA wide individual variations in uptake at each position were observed. The patterns and the levels of uptake in normal individuals were similar to the patterns and levels of uptake observed in patients with general paresis. 2. The percentage differences in uptake at symmetrical positions in normal individuals and in patients with general paresis were similar. This percentage difference rarely exceeded 10%. In contrast with this patients with cerebral neoplasms, who showed an increased uptake at the site of the tumor, showed differences in uptake from 12 to 50%.

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Seminal Amino Acid and Carbohydrate Pattern of Bulls with Normal and Abnormal Testes Function.* (19771)

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It has been adequately demonstrated by Mann and coworkers(1-4) that elaboration of fructose by the accessory sex organs ceases after castration and that it is readily resumed following the administration of testosterone. We were able to confirm and extend their findings(5,6). In a series of transfers of sperm from normal seminal plasma to plasma of castrate animals treated with testosterone and other steroids, it was shown by us(5) that the rate of fructose utilization decreased by as much as 70% even though the seminal fructose level was within or above the normal range. This justifies the speculation that the composition of seminal plasma obtained from such animals differs from that of the intact animal either because one or more constituents are lacking or because of a shift in the quantitative relationship of some of the plasma components. Thus, it was desirable to more thoroughly characterize the nutrients in seminal plasma elaborated under various physiological conditions.

I. *Chromatography of seminal carbohydrates.* It also became apparent that as far as the carbohydrate pattern of seminal plasma is concerned, the chemical methods used have not been adequate. While Mann and his coworkers(1-3) demonstrated that the seminal sugar is not glucose but fructose, they also stated that other sugars may be present in minute quantities. Since, to our knowledge, no attempt has been made to verify these findings by more sensitive methods such as paper partition chromatography, it seemed pertinent that this procedure be employed.

Experimental. The semen or seminal plasma was extracted with 80% ethanol and the precipitated proteins were removed by centrifugation. The supernatant extract was reduced to dryness at 50°C and the dry residue diluted with 10% isopropyl alcohol to one-half the original volume. This extract concentrate was chromatographed in one dimension using the method outlined by Patton(7).

It was evident that only one sugar, namely fructose, was found in the semen of these bulls regardless of whether they were normal, vasectomized, or castrated and restituted with testosterone. It was also evident that the separation of the various sugars on the papergram is specific and critical whether this was done with mixtures of pure sugars or when such sugars were singly or in mixtures incorporated with the seminal plasma to be chromatographed. The addition of glucose to a semen sample containing fructose resulted in the appearance of 2 definite spots, glucose always being in the lower position. The concentration of sugars in spots obtained on paper following the development with either the Seliwanoff reagent or aniline hydrogen phthalate was measured with a Densichron. We were able to correlate reasonably well the fructose values obtained by chromatography with results of standard chemical analyses.

II. *Chromatography of free amino acids in bull semen.* Little is known with respect to the free amino acids of seminal plasma. Tyler and Lord Rothschild(8) offered ample evidence that amino acids play an important role in the physiology of the sex organs. For instance, they found that certain amino acids increase the life span of sperm of the sea urchin but are not used up metabolically in this relation. Lorenz and Tyler(9) showed that glycine afforded protection to sea urchin sperm against damage from ultraviolet and visible light. It has been demonstrated that the metabolism of prostatic amino acids is also influenced by testicular hormones as has been shown to be the case with seminal sugars, for Marvin and Awapara(11) found a striking variation in concentration of free amino acids in the prostate of the intact rat as compared

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* This work was supported in part by Ciba Pharmaceutical Products, Summit, N. J., Armour and Co., Research Division, and the Bureau of Dairy Industry, Agricultural Research Administration, Department of Agriculture.

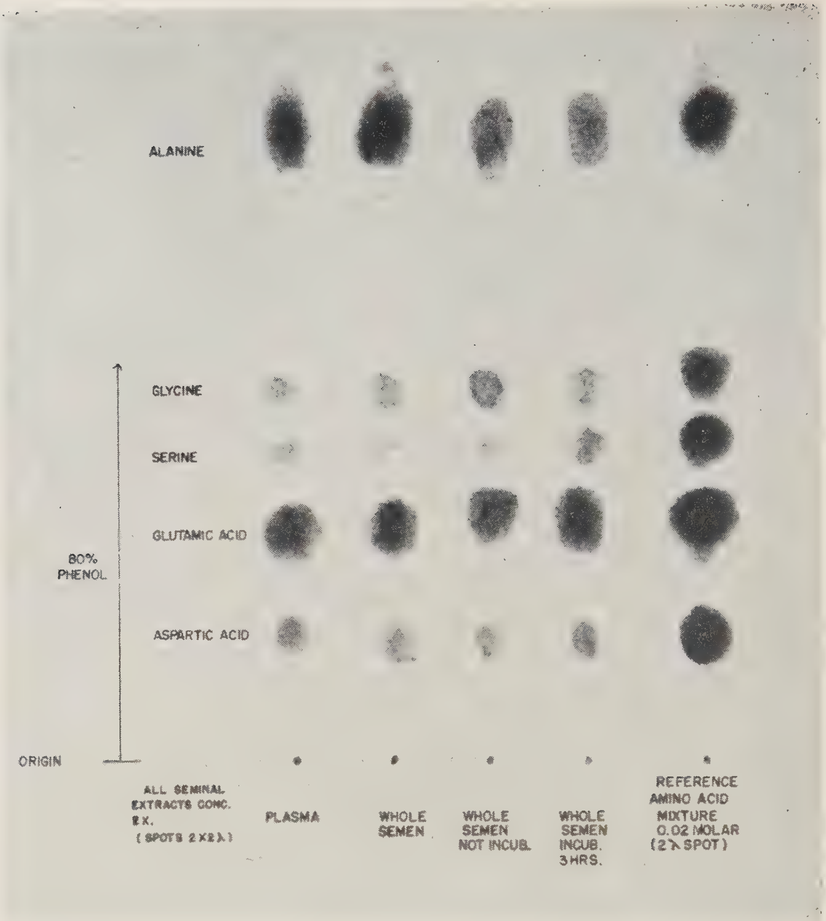

FIG. 1. Paper chromatogram of free amino acids in normal bull semen.

with the castrated animal. They further observed that androgen therapy caused a return of prostatic amino acids but the normal distribution pattern was not attained. Awapara(12) later indicated possible pathways of amino acid metabolism in the rat prostate. Thus, it seemed pertinent to examine the free amino acid pattern of semen from normal bulls and from animals which had been subjected to various experimental alterations of the reproductive system.

Experimental. A. *Free amino acid content of normal bull semen.* Seminal plasma or whole semen was extracted by a method similar to that used for the carbohydrates(13). The extract concentrate was chromatographed in 2 dimensions according to the method of Williams and Kirby(14) for qualitative estimation of free amino acids. Since separation of these amino acids was subsequently accomplished in one dimension using 80% phenol this was used thereafter.

Fig. 1 shows a typical one dimensional chromatogram of the 5 free amino acids found in normal bull semen. In the order of decreasing concentration they are glutamic acid, alanine, glycine, serine, and aspartic acid. The last column illustrates the separation of the acids from a .02 molar reference amino acid mixture. The concentration of any one of the 5 amino acids found was about the same in plasma and in whole semen. When the latter was incubated for 3 hours in order to permit metabolism of fructose by sperm, none of these amino acids was apparently utilized. This parallels the observation of Tyler *et al.*(8).

Densitometric measurements of amino acids were employed using a photoelectric scanning

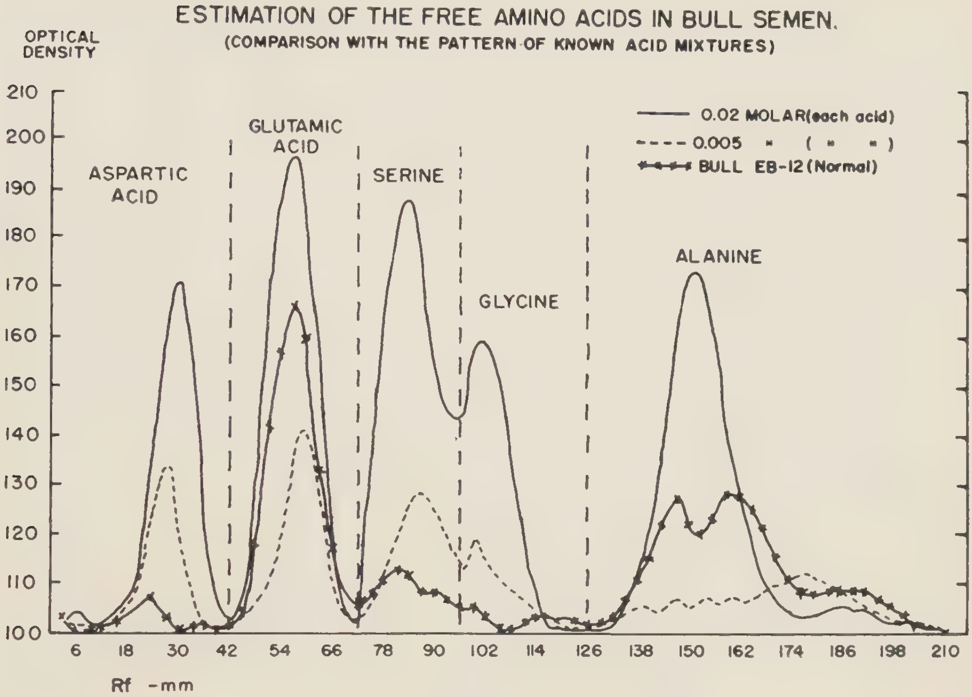

FIG. 2.

device, the Welch Densichron. The chromatographic pattern of the amino acids present in plasma specimens was measured using a paper guide, developed by Patton and Chism(15) by means of which ½ inch strips cut through the vertical axis of a finished chromatogram may be moved across the scanning probe in 3 mm increments with a 3 mm light aperture for illumination so that the strip can be studied in its entirety. The results of a typical measurement can be seen in Fig. 2. The optical densities of the various spots are plotted against the R_f in millimeters. The solid line represents the .02 M concentration of each acid while the dash line shows the concentration of the same acids at .005 M. The cross line indicates the pattern found in normal bull semen with glutamic acid showing the highest peak followed by alanine which gives a more diffused peak for both the seminal extract and the reference amino acids. The area enclosed by the curves represents the total concentration of a given amino acid in a spot. Although planimetric computation of such areas is more reliable, it is too tedious to be practical. It has been shown, however, that under properly controlled conditions the peak-density method is considerably faster and affords a reasonably accurate means of amino acid quantitation (15). After preparing standard curves for each individual amino acid the peak-height density values were translated into micromoles per 100 ml of fluid.

B. *Free amino acid content of accessory sex organ fluids in the normal bull.* In order to determine the site of production of the free amino acids found in bull semen, the seminal vesicles, ampullae of the vasa deferentia, and the epididymi of 4 normal bulls were obtained at slaughter. It was not difficult to secure up to 10 ml of seminal vesicular fluid and up to 1 ml of fluid from the ampullae. It was, however, impossible to express a sufficient amount of prostatic fluid without contamination from the tissue which surrounds this organ. The results of the estimation of amino acids are shown in Fig. 3. It is apparent that seminal vesicular fluid had an amino acid pattern similar to that found in seminal plasma with the exception that alanine and not glutamic acid was predominant. The ampullar fluid, however, showed a much higher

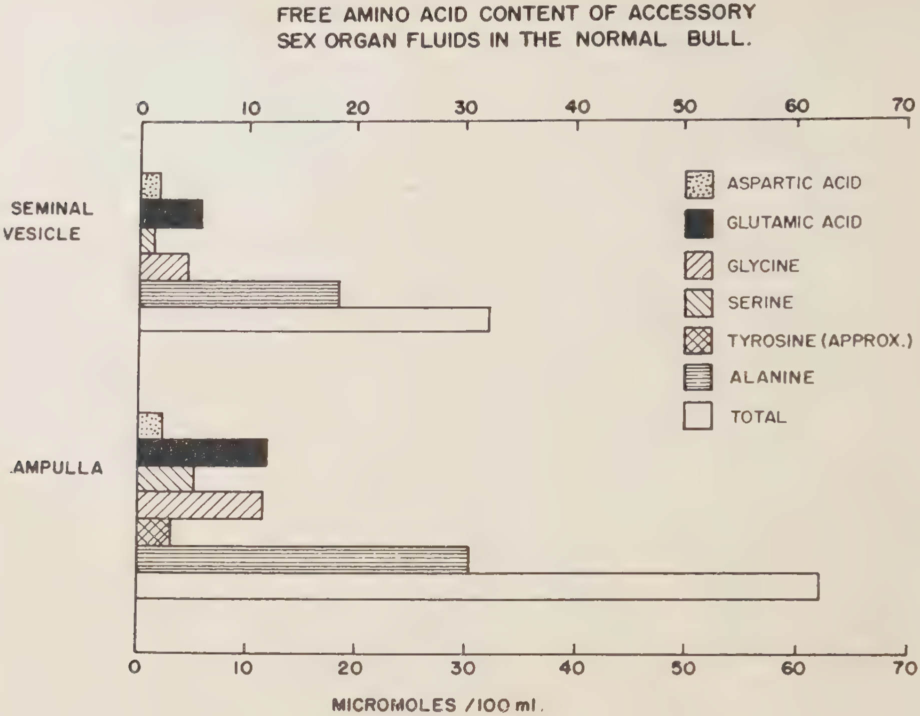

FIG. 3.

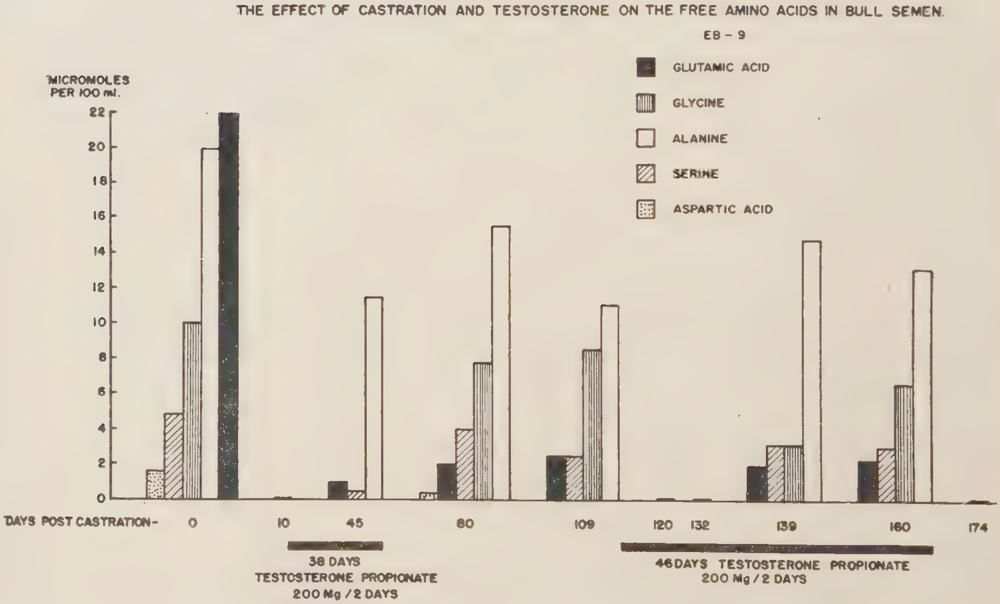

FIG. 4.

concentration in total acids per unit volume; a sixth amino acid, tyrosine, was found in 3 out of 4 specimens although in small quantity. It was not possible to obtain fluid from the epididymi in quantities sufficient for analysis.

C. *The effect of castration and testosterone*

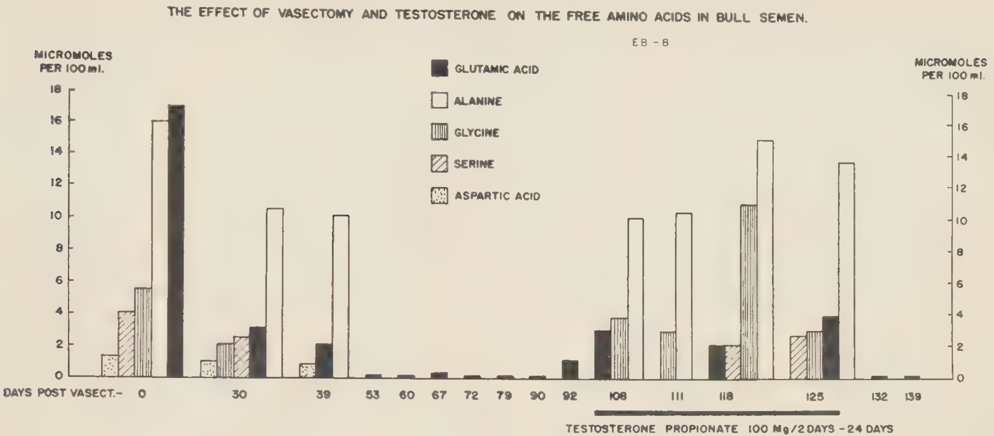

FIG. 5.

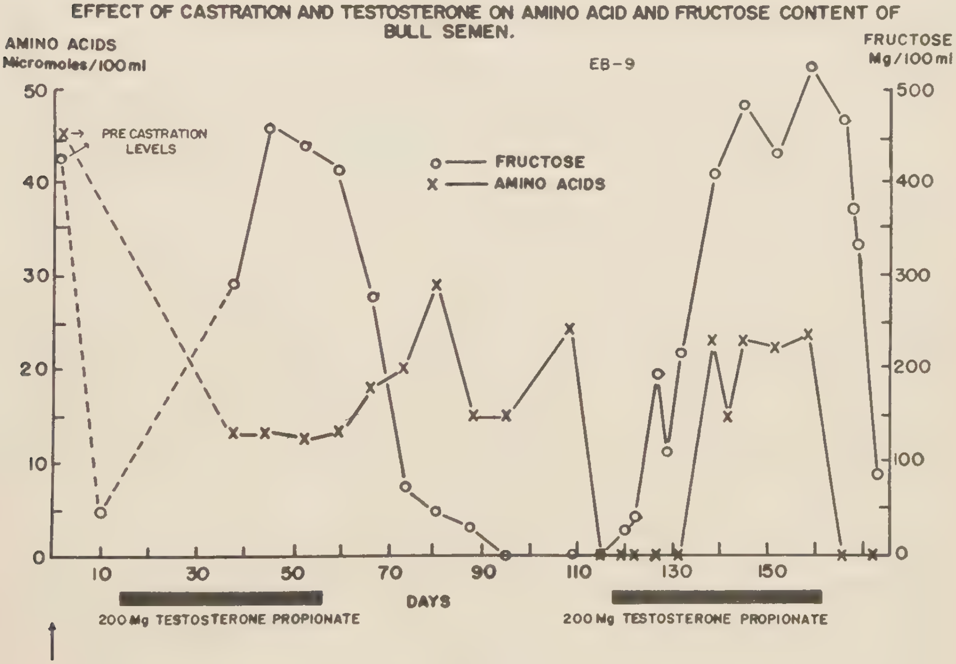

FIG. 6.

on the free amino acids in bull semen. Fig. 4 shows the effect of castration and subsequent testosterone therapy on the amino acids in normal bull semen. The 5 free amino acids disappeared within 10 days after castration. Administration of testosterone propionate (Perandren, Ciba) at 200 mg every 48 hours caused a return, within 3 weeks of alanine, glutamic acid, and serine at considerably lower levels. Glycine returned at a much slower rate while aspartic acid remained absent. Glutamic acid suffered the greatest loss whereas alanine was less affected. Although steroid therapy was discontinued after 38 days of treatment, the amino acids remained at a sub-normal level for another 51 days; thereafter, none was found. Upon the resumption of testosterone therapy for 17 days, a return of some of the amino acids was noted, but again, in no instance did this treatment result in a restitution to normal levels. The rapid loss of amino acids occurring when

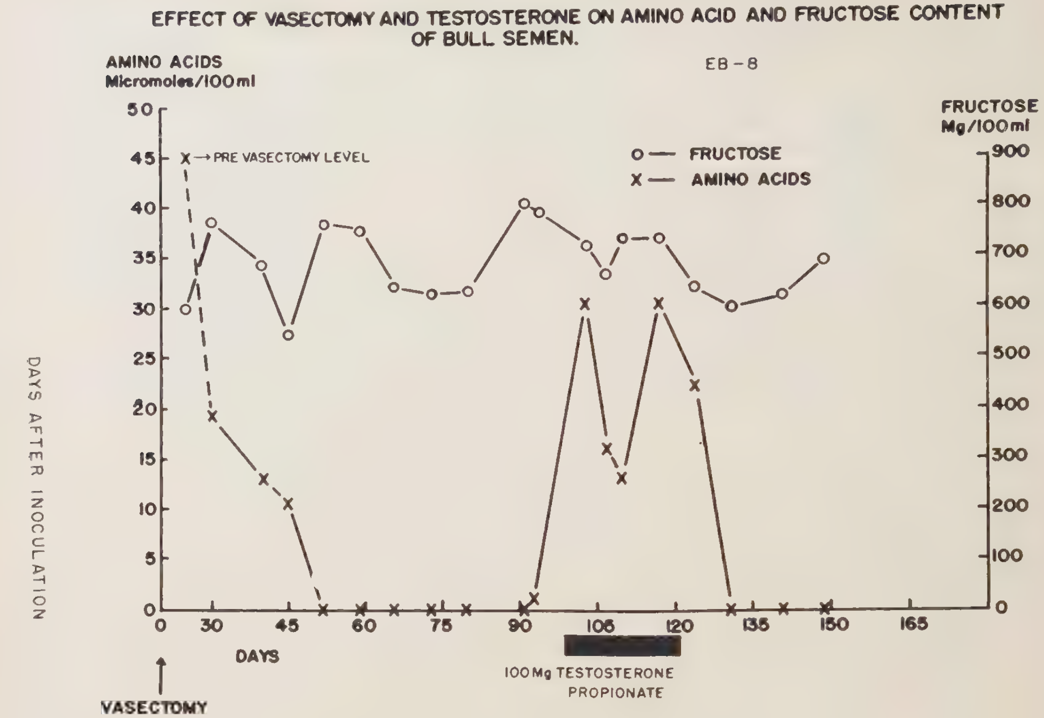

FIG. 7.

the steroid was again withdrawn is in contrast to the sluggish response of amino acids to earlier testosterone administration and withdrawal.

D. *Effect of vasectomy and testosterone on the free amino acids in bull semen.* A rather unexpected response was obtained following vasectomy. As seen in Fig. 5 the semen of this bull showed a normal amino acid concentration before vasectomy while 30 days later a marked decrease was noted; glutamic acid showed the greatest loss. Fifty-three days after vasectomy and for 2 months thereafter the seminal plasma contained no detectable amino acids. (In 2 instances trace amounts of glutamic acid were found). Testosterone propionate (Perandren, Ciba) was then administered at 100 mg every 48 hours. Seven days later the amino acids began to reappear. They returned to almost normal levels with respect to alanine and glycine but not with respect to glutamic acid. The amino acids disappeared from the plasma a week after testosterone was withdrawn. As demonstrated in Fig. 6 fructose disappeared rapidly after castration and returned with equal rapidity upon testosterone therapy. The amino acids behaved similarly but with delayed response, especially during the early post-castration period. Certainly there is a similarity in behavior of these seminal components in the castrate. Since in the vasectomized animal the testes remain *in situ* and since the testicular hormones continue to be elaborated, it could be expected that no loss in seminal sugar will occur after vasectomy. This is actually the case as shown in Fig. 7 which demonstrates that the seminal fructose remained high. Curiously, however, the free amino acids were absent by the 50th day after vasectomy. On the other hand, the administration of testosterone, while having no effect on the fructose level, resulted in a return of amino acids to near normal levels. The amino acids disappeared again when the steroid was withdrawn. Further experimentation is in progress in an attempt to explain this behavior.

Summary. 1. Methods of characterization and estimation by paper partition chromatography of carbohydrates and free amino acids in semen and accessory sex organ fluids of the bull are described. 2. Fructose was the only sugar found in semen and in fluids expressed from the accessory sex glands. The measurement by paper chromatography agrees well with the results of chemical methods. 3. The five amino acids found in free form in semen were glutamic acid, alanine, glycine, serine, and aspartic acid. They are apparently not utilized by spermatozoa during the process of fructolysis. 4. This pattern was similar in fluids obtained from seminal vesicles and the ampullae vasa deferentia except that alanine, not glutamic acid, was predominant. A trace of tyrosine was found in ampullar fluid. 5. Seminal fructose and amino acids were completely lost after castration. While testosterone therapy caused a rapid return of seminal fructose, the normal levels of amino acids were only partially restored. 6. Vasectomy, contrary to expectations, resulted in a total depletion of seminal amino acids even though the testes remained *in situ*. That the testes continued to function normally with respect to androgen secretion was borne out by the fact that masculine behavior, sex drive and the level of seminal fructose were not affected by vasectomy. 7. Treatment with testosterone propionate, however, restored seminal amino acids to significantly higher levels. Seminal fructose remained unchanged.

The authors gratefully acknowledge the cooperation of Dr. H. J. Hill, Artificial Insemination Unit and Dr. A. R. Patton, Department of Chemistry, Colorado A & M College, as well as the generosity of F. E. Houghton, Ciba Pharmaceutical Products, Summit, N. J., in supplying Perandren (testosterone propionate).

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Effect of Castration and Steroid Therapy on Seminal Plasma with Respect to Fructose Utilization by Normal Bull Sperm.* (19772)

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The demonstration by Mann(1) that fructose and not glucose is the glycolyzable reducing sugar present in mammalian semen stimulated considerable interest in sperm physiology from a new and different viewpoint. Seminal fructose is elaborated by the accessory male reproductive organs under the control of the testis hormones and represents the principal nutrient substrate for the metabolic activity of spermatozoa. This was shown in various animals by Mann and coworkers(2-7). These authors were able to show that fructose

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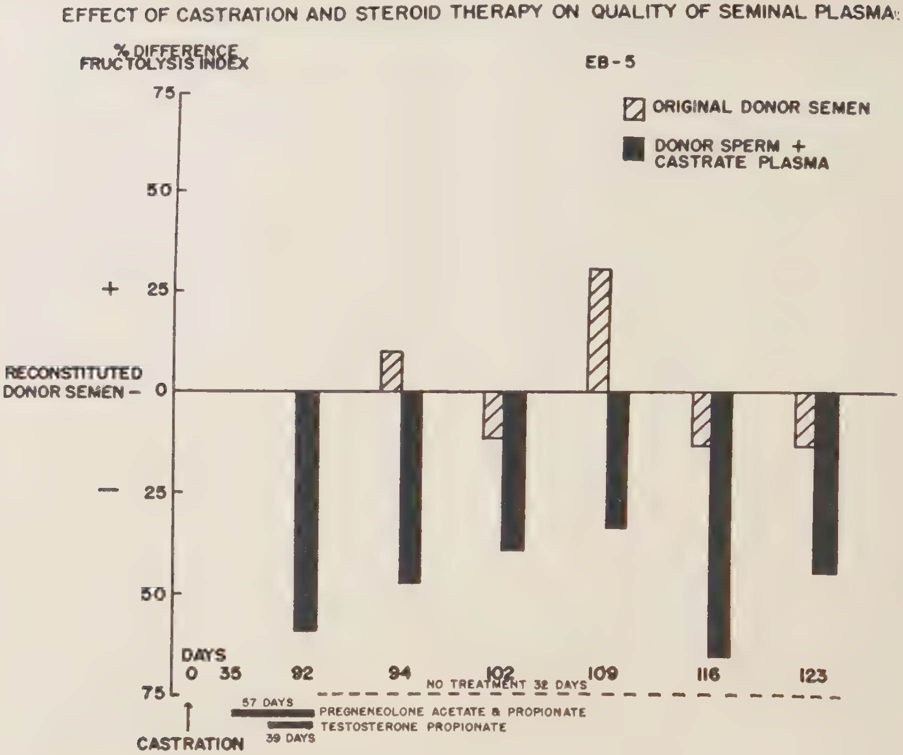

FIG. 1.

disappears from seminal plasma as well as from the accessory sex organs after castration and that implantation or injection of testosterone restores the normal fructose levels.

Gassner *et al.*(8,9) confirmed and extended these findings with respect to the utilization of fructose by spermatozoa, the effects of castration and restitution with androgenic and estrogenic steroids on seminal fructose of the bull, and the effects of gonadotrophins on the fructolysis index of sub-fertile breeder bulls.

Although the restitution of the accessory sex organs of the castrated bull is rapid and apparently complete, relative to size of the glands and secretion of fructose and citric acid, the question arises: Is the seminal plasma of the hormone stimulated castrate similar in composition to that produced by the intact animal and is it capable of supporting normal sperm metabolism? It is the purpose of this paper to report on the investigation of this problem.

Experimental. The procedure and results of a preliminary trial(9) were briefly as follows. The analytical methods were those reported by Gassner *et al.*(8).

A semen sample obtained from a normal bull was separated into 3 aliquots. One aliquot was retained as a control. Two aliquots were separated into plasma and sperm by centrifugation. The sperm residues were washed twice with phosphate buffer solution. One sample of reconstituted semen was obtained by combining one sperm residue with normal plasma in original proportions, while a second sample was obtained by combining the sperm residue with plasma of high fructose content from a bull which had been orchidectomized 3 months previously and was under treatment with pregnenolone and testosterone in an attempt to alleviate castration changes (9, p. 194). These sperm transfers were performed when the fructose content of the castrate plasma ranged from 600 to 800 mg %. The normal semen aliquot and the two reconstituted semen samples were then analyzed for fructose content, sperm concentration, and rate of fructolysis as outlined

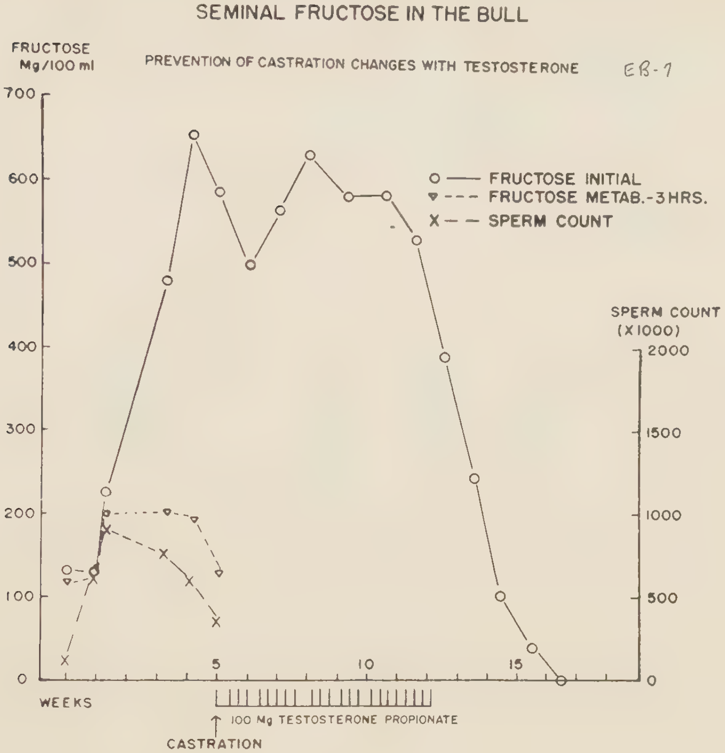

FIG. 2.

by Mann (11). In 6 consecutive weekly trials, it was found that the fructolysis index of the donor sperm-castrate plasma was 35 to 68% less than that of the donor sperm-normal plasma. (Fig. 1).

In subsequent work, the question arose as to whether semen from different individuals may be intermixed with compatibility to allow normal metabolism of fructose by spermatozoa. Semen samples from 3 normal bulls were analyzed individually and in a mixture of equal proportions for sperm concentrations, fructose content, and rate of fructolysis. The mixed semen sample was separated into plasma and sperm fractions by centrifugation. The plasma mixture and sperm mixture were then combined in original proportions. The fructolysis index† for each bull was found to be 2.83, 3.50, and 2.80, resp., with 3 hr incubations, for an average of 3.17. The index for an aliquot of the whole semen mixture was 3.17. The index for the reconstituted semen mixture (sperm mixture plus plasma mixture) was 3.50. Thus, there was no change in activity due to mixing of semen from different donors. It was also demonstrated that the procedure employed in sperm transfer has no effect on the fructolysis index.

It appears that, since normal sperm were not able to utilize fully the fructose in seminal plasma of castrates, the chemical composition of plasma from castrates restituted with certain steroids differs from the seminal plasma produced by the intact bull in some manner. These observations further suggest that the normal testis may elaborate hormonal substances other than testosterone and estrogen or that the epididymal fluid may contribute nutrients which are not furnished by the accessory sex organ fluids, all of which are required by spermatozoa for the maintenance of normal fructose metabolism. In an attempt to shed further light on this problem the following experiments were conducted.

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† (Fructolysis index: mg. fructose metabolized in 3 hr/10^9 sperm cells).

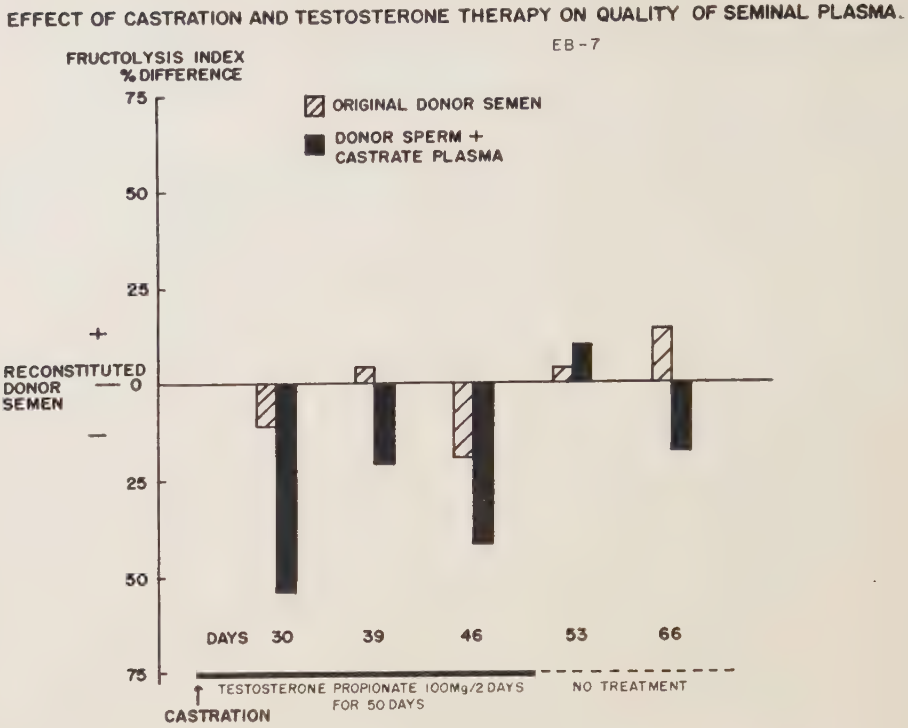

FIG. 3.

1. *Effect of castration and maintenance with testosterone on seminal plasma quality.* A normal bull was castrated and received, on the same day and every 2 days thereafter for 50 days, 100 mg of testosterone propionate (Perandren, Ciba). The seminal fructose level remained high until a week after testosterone was withdrawn whereupon it rapidly declined (Fig. 2). Normal bull sperm transferred at weekly intervals to plasma obtained from this castrate failed to utilize as much fructose as the sperm combined with normal plasma (Fig. 3).

2. *Effect of castration and restitution with testerone on seminal plasma quality.* A second normal bull was castrated. Supporting steroid therapy was delayed in order to allow the accessory sex glands to regress in size and function. After seminal fructose had dropped to zero, 200 mg of testosterone propionate (Perandren, (Ciba) was injected every 2 days. Seminal fructose returned rather rapidly and disappeared soon after the steroid was withdrawn. Following an 8-week rest period testosterone therapy was resumed with the result that the seminal fructose again rose to the precastration level. Upon cessation of the therapy, fructose again disappeared (Fig. 4). Seminal plasma of this bull was tested repeatedly to determine its ability to support the utilization of fructose by normal sperm (Fig. 5).

Two weeks after castration and before treatment with testosterone was begun, seminal fructose had declined to 27 mg %. When 400 mg % fructose were added, only 20% of the fructose present was metabolized by the donor sperm. Testosterone seemed to have an immediate effect on the seminal plasma since the rate of fructolysis was greater than that shown by the reconstituted donor semen. Upon withdrawal of the steroid, only 30% of the fructose present was utilized. The effect of resumption of steroid therapy is shown in that fructose was again more efficiently metabolized. Thereafter the seminal plasma quality seemed inadequate to fully support fructolysis by normal sperm. It should be pointed out that 5 months had elapsed since castration and that the response seen may indicate a loss in target organ re-

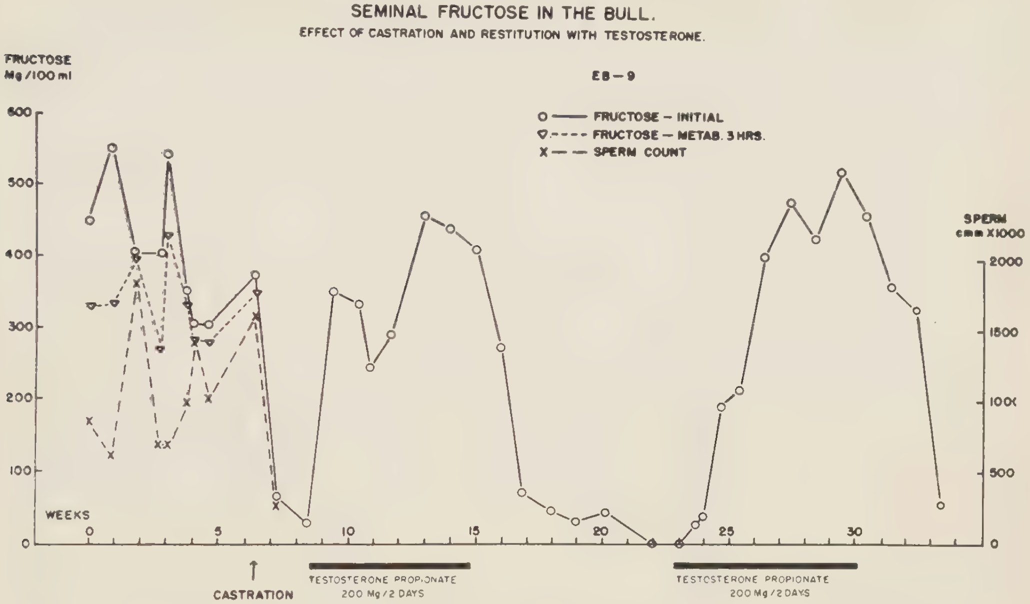

FIG. 4.

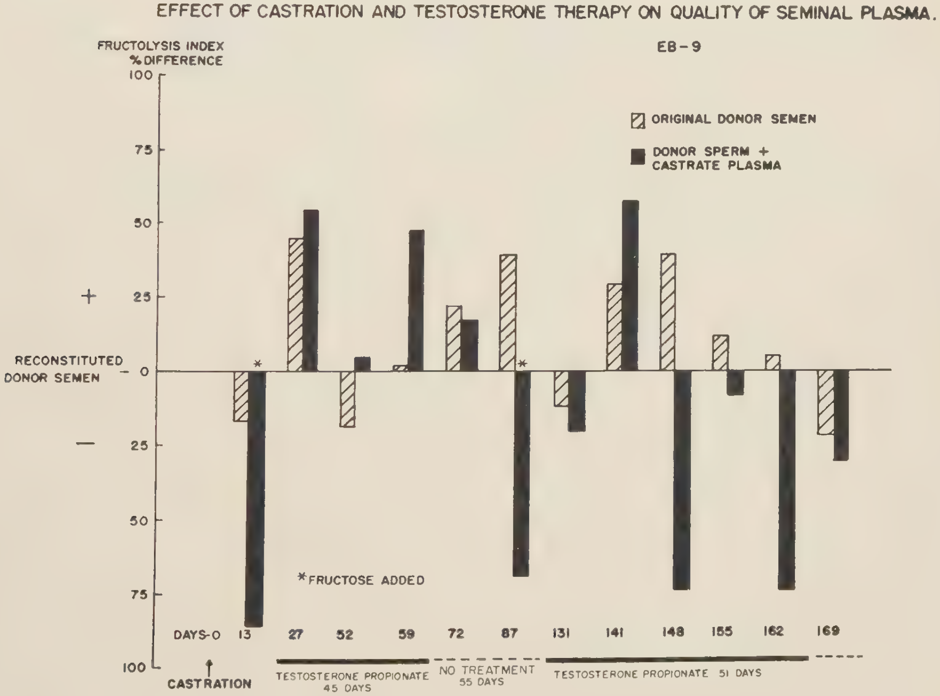

FIG. 5.

sponse as observed earlier(9).

3. *Effect of vasectomy and testosterone on the quality of seminal plasma.* Since in the vasectomized animal the testes remain *in situ*, the accessory sex glands remain under the trophic influence of the testis hormone and should produce a normal plasma. Therefore, it should behave in a similar fashion as normal seminal plasma deprived of sperm by centrifugation. To test this assumption vasore-

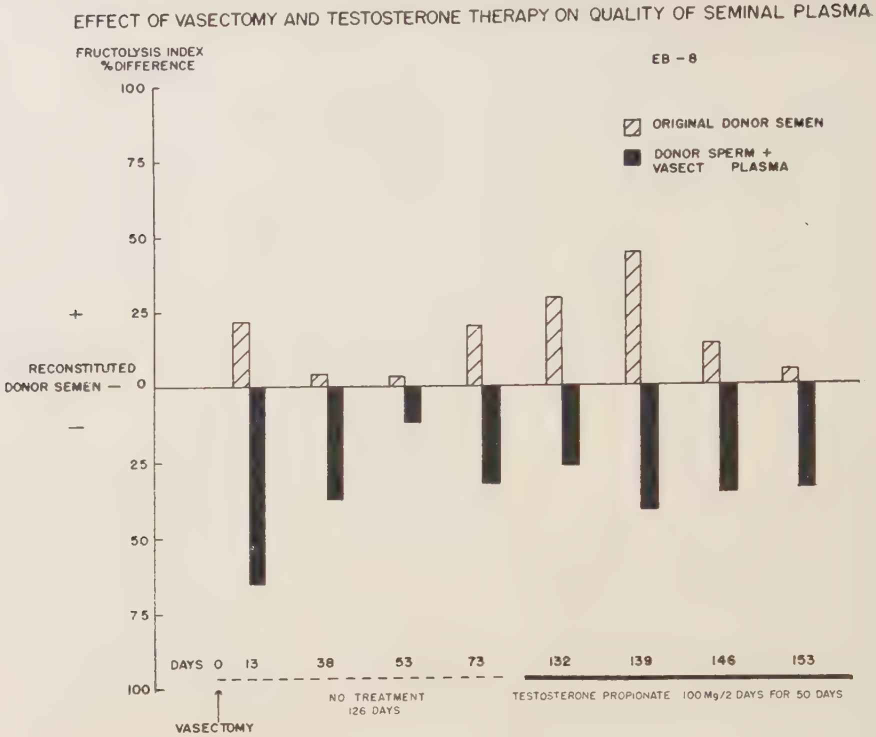

FIG. 6.

section was performed on a normal bull. The seminal plasma was analyzed for fructose concentration at weekly intervals. The fructose content was in no way affected by vasectomy nor was it changed when testosterone propionate was given for a period of over four weeks. Normal bull sperm were transferred repeatedly to plasma obtained from this animal. While during the first 2 months after vasectomy the degree of fructose utilization by normal sperm gradually improved, it remained below normal thereafter regardless of the fact that testosterone propionate was given (Fig. 6).

Discussion. At the moment it is impossible to draw any definite conclusions. It appears that testosterone cannot fully compensate for castration changes brought about in the accessory sex organs, particularly as far as the chemical composition of their secretions is concerned. The response shown by the vasectomized animal is puzzling. If the evaluation of the role of the governorship exerted by the testes over the accessory glands is correct, then there should be no difference in the metabolic behavior by normal sperm when reconstituted with either normal plasma or with plasma delivered by the vasectomized bull. A similar response to vasectomy was shown with respect to other seminal components, namely, the free amino acids(10). We have been able to show that the free amino acids disappear completely after vasectomy but return, at least in part, when testosterone propionate is injected. It has been further shown by us that in the normal bull the amino acid pattern of fluids obtained from the ampullae vasa deferentia is quite similar to that of the seminal vesicles. This justifies the tentative conclusion that vasectomy affects the functional status of the accessory sex structures.

Another series of bulls, vasectomized or castrated and receiving androgens and estrogens and mixtures thereof, are at the present time on trial. Information received so far from these experiments has confirmed the observation reported here.

Summary. 1. The method employed for

the separation of normal bull semen into plasma and sperm with subsequent reconstitution appears to have no detrimental effect upon the ability of the sperm to metabolize fructose. 2. Similar results were obtained when semen from three normal bulls was intermixed. 3. The immediate administration of testosterone propionate to a bull at the time of castration prevents the loss of seminal fructose resulting from castration. Seminal plasma from such an animal was not fully capable of maintaining the metabolism of fructose by normal sperm. 4. Seminal plasma of negligible fructose content from a bull in which castration changes were allowed to develop before testosterone therapy was begun failed to support a normal rate of fructolysis since only 20 per cent of added fructose was utilized by normal sperm. Testosterone therapy caused a marked increase in both seminal fructose and the utilization thereof. Subsequent withdrawal and resumption of steroid therapy produced similar results. Thereafter, in spite of continued treatment with testosterone, the seminal plasma of this castrate only partially supported fructose metabolism. 5. Vasoresection in a bull had no adverse effect on either sex drive or the production of seminal fructose. However, when normal sperm was transferred to seminal plasma from this bull, the utilization of fructose was, contrary to expectations, markedly reduced. This would indicate that vasectomy adversely affects the functional status of the accessory sex organs.

The authors gratefully acknowledge the technical assistance of Lois Lucas. Thanks are due F. E. Houghton, Ciba Pharmaceutical Products, Inc., Summit, N. J., for the generous supply of Perandren (testosterone propionate).

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Development of Electrical Activity in the Cerebral Cortex of the Albino Rat.* (19773)

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Rhythmical electrical activity appears rather suddenly in the cerebral cortex of the guinea pig during the seventh week of gestation(1,2). Various morphological and biochemical changes also occur at this particular stage of development in this animal(3,4). The present study is concerned with an attempt to determine the critical period, for the albino rat, at which the cortical neurons become functionally active, using the electrocorticogram as an indicator of this development.

Methods. About 50 albino rats of various ages from the newly born to the adult were studied. Ether, diallylbarbituric acid (25-50 mg/kg, I.P.), or d-tubocurarine chloride (0.2-1 mg/kg, I.P.) were used to immobilize the animals in order to permit direct recording of electrical potentials from the cerebral cortex. Artificial respiration was administered, by means of short bursts of air from a mechanical pump into a tracheal cannula, before curarization. (Cannulae suitable for the young animals were fashioned out of standard No. 20 hypodermic needles). As long as this ventilation was adequate, cortical electrical activity was not seriously affected even after complete muscular paralysis(5-7). Surgical procedures, *e.g.*, tracheal cannulation, exposure of the cortex, were performed under ether anesthesia. The entire procedure was carried out in a large tray maintained at about 37°C and the exposed skull or cortex was periodically moistened with warm mammalian Ringer-Locke's solution.

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A 3-channel, Offner D-C amplifier (based on a carrier-wave, chopper circuit) and operating ink-writer were used in conjunction with a battery-operated, differential type, cathode-follower input stage. The entire instrument therefore constituted a direct-coupled amplifier with a maximum sensitivity of about 250 μv per cm pen deflection, and a frequency response, uniform to within 10%, from 0 to about 40 cycles per sec, and falling off by 60% at 75 cps. The paper drive could be run at various speeds from 0.5 to 25 mm/sec.

The recording electrodes consisted of Ag-Ag Cl wires dipping into pipettes filled with Ringer's solution, and making contact with the tissue via cotton threads protruding from the pipette tips. Bipolar recording was used most frequently, with a large Ag wire inserted into the mouth for a ground electrode. Spurious changes of potential between these pairs of recording electrodes were routinely monitored by connecting the pair through a piece of Ringer-moistened filter paper. Control of artifacts due to movement of the cortex with respect to the electrodes was made by recording simultaneously from an active and a nearby surgically or chemically inactivated area.

Strychnine was applied to the cortex either in the form of a small crystal of strychnine sulfate or on a small piece of filter paper soaked in a 2% solution of that salt.

Results. During the first week after birth, the electrical activity detectable from the cerebral cortex was irregular, intermittent, and of relatively low amplitude. "Silent" periods were common. The frequencies tended to be of the order of several cycles per second. From the fourth day onward, local application of strychnine resulted in the appearance of spike potentials of the order of several tenths of a millivolt and about 0.5-1 sec. in duration. These usually developed within a few minutes after the application, and occurred, irregularly, several times per minute. By the seventh day, spikes so evoked were as large as one millivolt, and their durations less than 750 msec. They also occurred more frequently, *i.e.* once every several seconds.

Between the seventh and tenth days, a marked trend towards regularity, rhythmicity, and continuous activity was seen in the records. Silent periods were almost nonexistent by the tenth day. The amplitudes were often as large as several tenths of a millivolt, while the frequencies varied from several to about 20 per sec. Strychnine-induced spikes were now of still shorter duration—about 250-500 msec, and occurred more frequently—sometimes as often as several per sec.

The developments in the ECoG patterns subsequent to the tenth day were relatively minor. By the fourteenth day the amplitudes were somewhat larger, both with regard to spontaneous as well as strychnine-induced activity. Moreover, the duration of the strychnine spikes decreased to about 100 msec. The patterns observed at this age level were basically similar to those of the adult rat.

In a few cases, the entire surface of the cortex was explored for differences in the rate of development of electrical activity, but no significant differences were detected. In general, it was found that the largest amplitudes were obtained from the frontal and parietal regions.

In some cases, the cortex was locally inactivated, either by cutting a circle in the cortex about 2 mm deep, or by applying a piece of filter paper soaked in a 2% solution of Nupercaine (x-butyloxycinchoninic acid-γ-diethylethylenediamide hydrochloride). Within a few minutes, most of the electrical activity disappeared in that region.

Discussion. The fact that local application of strychnine to the cortex resulted in typical spike activity indicates that at least part of the spontaneous electrical activity described above arises from cortical cells(8). The lack of appreciable pickup from subcortical regions is also shown by the results of local inactivation of the cortex.

Many morphological changes occur in the rat cerebral cortex between the 5th and 10th days after birth. Sugita found that the thickness and volume of the cortex increased sharply during this period, while the number of cortical neurons per unit volume decreased rapidly, all three parameters suddenly approaching the adult levels on about the 10th day(9,10). He also observed that the nuclei and nucleoli of the cortical neurons suddenly reached their maximum size on this day. Furthermore, Nissl bodies first appeared on the 9th day and gradually became more abundant. Tilney(11) also found relatively little cellular or histological differentiation during the first week after birth and marked development during the second week. His observations of the behavior patterns of 5- and 10-day-old rats showed that little if any were of such complexity as to imply a cortical level of neural correlation. The exploratory and playful activity of the 15-day-old, however, could no longer be reasonably ascribed to infracortical levels of correlation. It is interesting to note that the appearance of continuous, regular, rhythmical electrical activity in our experiment coincides very closely with the end of the period of rapid cellular and histological maturation in the rat cortex. The spike potentials elicited by strychnine also approach their adult form soon after this time.

These results agree well with ECoG studies on kittens(12) and young rabbits(13,14). In the guinea pig cortex, it has been shown that the period of rapid morphological development occurs between the 41st to 46th days of gestation(3,4). The analogous period in the rat occurs between 7 to 10 days after birth. In the guinea-pig, however, well-developed, spontaneous, as well as strychnine-elicited, cortical activity appear suddenly on the 46th day of gestation, whereas none could be detected on the 45th day(2). The more gradual development of electrical activity in the rat, cat, and rabbit, beginning well before most of the cortical cells have undergone appreciable maturation, may be related to the multitude of new stimuli that impinge upon these postnatal animals resulting in the acceleration of the development of certain critical processes necessary for the appearance of such activity. In spite of this difference, however, the fundamental cortical developmental processes are essentially the same in both groups.

Summary and conclusion. 1. Electrocorticograms were recorded from albino rats immobilized with various drugs. 2. Spontaneous electrical activity was irregular, intermittent, and of relatively low amplitude during the first week after birth. 3. Local application of strychnine to the cortex resulted in large, slow, spike potentials in rats several days old. 4. The general trend in the spontaneous activity from birth to about 10 days of age was towards increasing rhythmicity, regularity, continuousness, and amplitude. The strychnine-induced spikes gradually decreased in duration, and increased in amplitude and frequency of occurrence. 5. The ECoG of the 10-day-old rat was already basically similar to that of the adult.

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Adrenal Insufficiency in Dogs with Normal Serum Na and Cl.* (19774)

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During the past few years the writers have studied an extensive series of adrenalectomized dogs through cycles of insufficiency and recovery while they were receiving various kinds of replacement therapy and fed a diet low in Na and Cl. During this study an opportunity arose to observe 5 animals which exhibited mild to very severe symptoms of adrenal insufficiency despite the fact their serum Na and Cl levels were well within the normal range. One other dog was also studied in which the serum Na and Cl remained unchanged from normal until a few hours before collapse, when, following the sudden onset of a moderate diuresis, the electrolytes decreased to low levels and severe symptoms appeared. Attention has been called to the ease with which adrenalectomized dogs can be maintained for long intervals free from signs and symptoms of insufficiency, but with serum Na and Cl concentrations characteristic of animals prostrate from adrenal insufficiency(1-3). The situation in which the animal may display marked symptoms and yet exhibit a normal serum electrolyte pattern is, insofar as the dog is concerned, an uncommon occurrence. Nicholson and Soffer(4) observed several such cases and attributed absence of decline in serum electrolytes to hemoconcentration and dehydration. Thus according to these authors "when hemoconcentration has become so marked that insufficient water is available for the excretion of electrolytes, the sodium and chloride of the plasma may be increased, and this fact may explain the apparent paradox occasionally observed of typical suprarenal insufficiency with high plasma sodium and chloride values."

Although the adrenalectomized dog rarely displays insufficiency symptoms with normal serum Na and Cl concentrations, certain other animal species commonly do so. For example, it has been reported that the opossum and marmot show increased plasma concentrations of Na and Cl even when the animals are practically moribund from adrenal insufficiency (5,6). This finding has been confirmed for the opossum(7). Hartman *et al.*(8) found that extirpation of the interrenal gland of elasmobranchs failed to induce changes in the serum Na and Cl. This author, together with Spoor(9), has described the separation of a Na retaining factor from adrenal cortical extracts which elevates the plasma Na of adrenalectomized animals but does not prevent the onset of insufficiency symptoms. The chemical nature of this factor remains undetermined. Finally, it seems not improbable that the Addisonian patient may occasionally succumb without exhibiting undue distortion of the plasma electrolyte pattern(10).

Results. Dogs 1-5, listed in Table I, although showing symptoms of insufficiency, nevertheless had normal or low normal serum Na and Cl. These animals had received various types of therapy while maintained on a low sodium and potassium diet and had been employed in bioassay studies involving use of various esters of desoxycorticosterone or concentrates of beef adrenal extract. The symptoms varied from mild (dogs 4-5) to severe (dogs 1-3, Table I). The dogs refused all food, showed marked depression and lassitude, were spastic and showed signs of weakness. The essential data regarding weight changes, arterial pressure and serum electrolytes are summarized in Table I. Only the control and terminal figures are tabulated. The methods used for all determinations and the diet employed have been referred to elsewhere(11).

Adrenalectomized dogs exhibiting insufficiency and with normal serum Na and Cl arouse suspicion that hypoglycemia might be present and thus account for some of the

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TABLE I. Adrenal Insufficiency in Dogs Exhibiting Minor Changes in Serum Na and Cl.

Dog No.			Body wt, kg	B.P., mm Hg	Blood urea N, mg %	Hb, g %	Blood glucose, mg %	Serum Na, mEq/1	Serum Cl, mEq/1	Serum K, mEq/1	Remarks
1	*		9.6	113	9	10.1	78.5	139	112.6	4.5	§
	†		8.3	68	‡	‡	‡	137	109.6	5.4	Spastic, lethargic, weak, refused rations
2	*		18.8	118	9.7	9.7	76	141.4	115	6.1	§
	†		18.1	52	120	21.4	85	144	114.5	10.2	Severe symptoms, spastic, weak, refused rations.
3	*		10.1	100	15	13.4	82	139.3	114.4	6	§
	†		9.2	52	48	17.5	67	136.4	110.2	8.3	Severe symptoms, spastic, weak, refused rations
4	*		16.1	112	14	15	75	145.4	118	4	§
	†		17	78	57.6	17.8	76	148	116	—	Refused rations, lethargic, weakness in hind limbs
5	*		17.5	130	13.5	15	70	148	112	4.2	§
	†		17.9	78	59.2	19.5	76.5	147	120	5.7	Fall in arterial pressure, marked hemoconcentration, rise in blood urea N
6	*		17.7	96	15.6	10.9	78.5	140	111.8	4.2	§
		11 a.m.	17.3	93	23	12	67.5	140	109.4	5.6	No symptoms
	†	11 p.m.	17.3	59	44.5	12.6	67.5	130.6	98.4	5.4	Refused rations, weakness in hind limbs, spastic

* Control. † Insufficiency. ‡ Not determined. § Normal.

symptoms. However, it seems unlikely that the blood glucose changes which occurred in the dogs (Table I) were of sufficient magnitude to be responsible for the observed symptoms of insufficiency. The changes were not marked and are above the hypoglycemic range. While it is true, as Thorn *et al.*(12) have pointed out, that the Addisonian patient and the adrenalectomized dog may show hypoglycemic symptoms at higher levels of blood glucose than does the patient or dog with intact glands, nevertheless the blood glucose figures for the animals listed are above the critical level even for adrenalectomized dogs.

Three of the animals listed in Table I (dogs 2, 3, and 5) showed severe hemoconcentration as indicated by the striking increase in hemoglobin concentration. The hemoglobin of dog 2 rose approximately 12 g % and the serum K was elevated to 10.2 mEq/1. The blood urea nitrogen registered 120 mg %. The hemoglobin concentration of dog 3 increased 4.1 g % and that of dog 5 by 4.5 g %. The evident dehydration and probable reduction in renal function could well account for the lack of the usual sodium and chloride diuresis in these animals and hence the maintenance of normal values for these ions in the serum. Dog 2 showed an extreme hyperkaliemia which was of sufficient degree to induce collapse owing to cardiac failure. We have repeatedly observed that serum K values in adrenalectomized dogs in excess of 8 mEq/1 may produce serious cardiac disturbances culminating in prostration of the animal. Thus, it seems evident that even in the dog, a species in which adrenal insufficiency is normally characterized by a sodium and chloride diuresis and drastic alterations in the serum concentrations of these electrolytes, it is possible in rare cases to have the onset of mild to severe symptoms with little or no apparent deviation from normal of the serum Na and Cl. Moderate renal retention of K was evident in all cases and was especially prominent in 2 animals (Nos. 2 and 3, Table I).

Dog 6 is an interesting case since the Na and Cl changes given in Table I occurred within a 12-hour period. The animal had been maintained for 3 days on 0.25 mg of the water soluble glucoside of desoxycorticosterone, and during this interval the serum electrolytes had shown no deviation from the normal pattern. At 11 A.M. on the day he developed symptoms of insufficiency, the blood pressure and blood constituents were normal and the dog appeared free from symptoms. However, by 11 P.M. marked symptoms had appeared: he had refused all food, was depressed, spastic and weak in the hind limbs. The activity and vigor which were so pronounced 12 hours earlier had disappeared. Blood samples were taken at this time and the results of the analyses are given in Table I. During the 12-hour interval (11 A.M. to 11 P.M.), the serum Na and Cl declined to 130.6 and 98.4 mEq/1, respectively. The sharp drop in serum electrolytes was accompanied by a moderate diuresis after which symptoms appeared.

Summary. A group of 5 adrenalectomized dogs of a large series studied, developed typical signs and symptoms of adrenal insufficiency in the absence of noteworthy alterations in the serum Na and Cl values. Hypoglycemia was not present in any of the animals but during insufficiency dehydration and hemoconcentration were evident in 3 of them as indicated by marked increases in hemoglobin. In view of the inspissation of the blood in these dogs it seemed probable that sufficient water was not available for elimination of the electrolytes. This is the interpretation originally given by Nicholson and Soffer(4) to account for the death of their salt-injected dogs. However, a comparison of the hemoglobin concentrations of 8 adrenalectomized dogs exhibiting insufficiency with hyponatriemia (avg. 128 mEq/1) with the hemoglobin increases shown by the 5 animals discussed here, reveals that the hemoconcentration as gauged by hemoglobin changes was not more severe in the latter group. It is unfortunate that plasma volume determinations were not made. However, the drastic decline in arterial pressure and evident weakness of the animals indicated that defective functioning of the vasomotor system was probably the immediate cause of the symptoms. The hyperkaliemia of one dog was of sufficient magnitude to induce cardiac symptoms. The retention of a normal serum electrolyte pattern during insufficiency in these few exceptional dogs is reminiscent of the condition normally occurring in adrenal insufficiency in such animals as the opossum, marmot hamster (13) and certain elasmobranchs.

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Intestinal Absorption of Histidine as Influenced by Tryptophan in the Rat.* (19775)

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It has been shown that amino acids have to be available simultaneously and in proper relative concentrations for optimal utilization in the tissues(1). The first step in the utilization of dietary amino acids is the intestinal transfer to the body proper and therefore the speed and rate of this transfer may significantly influence the simultaneous availability of the amino acids(2). There is only little information available on the mechanism of amino acid absorption. Some data have been published in the literature which suggest that the absorption of amino acids is not a process of simple diffusion, but that some other mechanisms probably some "accelerating factors" may be also involved(3). Further it has been shown by several authors that amino acids are absorbed selectively at varying rates from the gastrointestinal tract. It was the purpose of the present study to determine how the absorption of an amino acid, such as histidine or tryptophan, is influenced by the presence of other amino acids in the intestinal tract.

Method. The Cori technic(4) was used to investigate the absorption of amino acids from the gastrointestinal tract of adult male Sprague-Dawley rats. The rats were fed the amino acid solutions via stomach tube. L-tryptophan and dl-phenylalanine were fed as such, and L-histidine and L-arginine were fed as the monohydrochlorides. All of the amino acid solutions were adjusted to a pH of 7.0-8.0 before feeding. After a predetermined absorption period, the residual amounts of histidine and tryptophan which remained in the gastrointestinal tract were colorimetrically determined(5,6). The absorption coefficients were computed on a basis of body surface area rather than of body weight in an attempt to obviate any variability due to differences in the weights of the animals used.

For the determination of histidine Lang's modification of the Pauly-Weiss method was used(5), by which a red color is developed when histidine is treated in alkaline solution with freshly prepared diazotized sulfanilic acid. The only other amino acid giving this color under these conditions is tyrosine, which was completely removed by precipitation with $HgCl_2$. The test for tryptophan is based on the Voisenet-Rhode reaction(6), by which tryptophan specifically produces a blue color in an acidic medium of p-dimethylaminobenzaldehyde. In preliminary control experiments it was shown that the presence of tryptophan in the concentrations used did not interfere with the determination of histidine. Similarly, the color test for tryptophan was not modified by the presence of histidine.

Results. Table I lists the mean values of the absorption coefficients for histidine and tryptophan ± the standard deviations of the mean. Their absorption rates appear to depend to a certain limit on the concentration in which they were fed. The quantity absorbed increased with increasing concentration of the solution fed from 24 till 50 mg/7.5 ml. Greater concentrations, *i.e.*, 60 to 75 mg/7.5 ml, did not increase further the absorbed quantity of histidine or tryptophan. The data of the table show that the absorption rates of histidine and tryptophan did not differ statistically when each was fed by itself. The results of the experiments wherein histidine and tryptophan were fed simultaneously show that *the presence of l-tryptophan significantly decreases the rate of absorption of histidine from the gut.* This is seen clearly following the feeding of a solution containing 40 to 75 mg each of histidine and tryptophan in 7.5 ml. When histidine was present alone in the gut in this concentration, the absorption coefficient of histidine ranged from 35.7 ± 8.0 to 43.4 ± 7.1. When, however, both histidine

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TABLE I. Absorption Rates of Histidine and L-Tryptophan (15-minute absorption period).

Amino acids fed	No. of animals	Amount fed (mg/7.5ml) l-tryptophan	L-histidine	dl-phenylalanine	L-arginine	Absorption coef. (mg absorbed/100 cm²/hr) l-tryptophan	histidine
l-t†	1	30				*12.7	
	1	50				*43.2	
	1	75				*41	
h†	5		24.3				15.5 ± 4.6
	6		40.5				35.7 ± 8
	4		60.8				43.4 ± 7.1
l-t + h	5	30	24.3			24.8 ± 6.3	9.6 ± 5.1
	5	50	40.5			37.3 ± 8.2	12.9 ± 6.5
	4	75	60.8			47.5 ± 4.2	6 ± 3.1
l-t, h, and dl-phenylalanine	2	30	24.3	30		26.2 ± 1.7	4.3 ± 1.3
	2	50	40.5	50		41.1 ± 1.3	10 ± .5
	2	75	60.8	75		44.2 ± 3.8	3.5 ± 3.5
l-t, h, dl-phenylalanine, and arginine	2	30	24.3	30	24.8	26 ± 3	5.3 ± 5.3
	2	50	40.5	50	41.4	37.6 ± 2.5	1.1 ± .5
	2	75	60.8	75	62	35 ± 7.2	0 ± 0
h, dl-phenylalanine, and arginine	2		24.3	30	24.8		21.1 ± 3.1
	2		40.5	50	41.4		30.2 ± 8.6
	1		60.8	75	62		13.9

* Feeding of identical concentrations of l-tryptophan followed by 10, 20, and 30 min. absorption periods resulted in similar absorption data.

† t = tryptophan; h = histidine.

and tryptophan were present coincidentally in this concentration, the absorption coefficient of histidine was decreased to 6.0 ± 3.1 to 12.9 ± 6.5. *The absorption rate of l-tryptophan does not appear to be affected by the simultaneous presence of histidine, dl-phenylalanine and arginine in the gut.* The presence of dl-phenylalanine and arginine did not show any significant effect on the absorption of either histidine or tryptophan.

Discussion. Our results support further the assumption of some authors that a competition may exist between amino acids themselves or between amino acids and chemically similar compounds for the selective processes of absorption or concentration. In regard to this, Pitts(7) has shown that a competition exists between alanine and creatine for reabsorption by kidney tubule cells. Creatine may be regarded chemically as a substituted methyl glycine. The *in vitro* studies of Christensen and Streicher(9) indicate also that there may be a competition between alpha-amino acids for specific receptor groups in the tissues. Orten *et al.*(8) found also "alterations in the absorption rate of individual amino acids in the presence of other amino acids."

There is no satisfactory explanation yet for the amino acid imbalance which occurs in animals fed certain amino acid mixtures or proteins. It is possible that an amino acid imbalance may be a result of the differential absorption of amino acids from the gastrointestinal tract. The absorption of proper relative concentrations of the essential amino acids may be hindered by the presence of excessive amounts of some other amino acids. This problem is being presently further investigated.

Summary. The presence of l-tryptophan decreases the rate of intestinal absorption of L-histidine in the rat. The absorption rate of l-tryptophan does not appear to be affected by the simultaneous presence of histidine. Dl-phenylalanine and L-arginine do not seem to affect significantly the absorption of either histidine or tryptophan.

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A Critique of Biological Activity of L-Lyxoflavin.* (19776)

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Pallares *et al.*(1) claimed that lyxose was isolated from human myocardium treated with cobra venom. This group later reported(2) the isolation of lyxoflavin from human myocardium and postulated the release of lyxose by the enzymes of cobra venom. Gardner *et al.*(3,4) and Heyl *et al.*(5) subsequently described syntheses of L-lyxoflavin. Gardner *et al.*(3,4) failed to obtain lyxose from synthetic L-lyxoflavin treated with either cobra venom or Russell's viper venom. It was then reported that L-lyxoflavin has vitamin activity for the rat fed a diet containing 0.5% dessicated thyroid(6,7), and growth promoting activity for the chick(8).

Snell and Strong(9) have shown that other stereoisomers of riboflavin, namely the d- and l-arabityl analogs, enhanced the growth response of *Lactobacillus casei* to limiting amounts of riboflavin although these are devoid of activity when assayed in the absence of riboflavin. Other workers(10,11) have demonstrated that the d-xylityl and l-arabityl analogs of riboflavin have limited effectiveness in replacing riboflavin in rats receiving a riboflavin deficient ration. We wish to report our observations on the similar behavior of L-lyxoflavin in the nutrition of *Lactobacillus casei*, the rat and the chick.

Microbiological experiments. L-Lyxoflavin was tested for riboflavin activity using the assay of Snell and Strong(12). The results are shown in Fig. 1. It is apparent that lyxoflavin shows little activity in the absence of added riboflavin. The failure of the higher levels of lyxoflavin to give increased growth indicates that the slight activity of lyxoflavin is not due to contamination with riboflavin. However, in the presence of 0.10 γ of riboflavin, lyxoflavin approximates the activity of riboflavin at levels of 0.02 to 0.10 γ. At levels of 150 γ or more, lyxoflavin inhibits the response of the microorganisms to 0.10 γ of riboflavin. When these results were completed, Bruins *et al.*(8) stated without detailing the data that lyxoflavin stimulated the growth of *Lactobacillus casei* in the presence of limiting amounts of riboflavin and acts as an antimetabolite at higher concentrations.

Rat experiments. Emerson and Folkers(7) found that lyxoflavin was without activity in a prophylactic rat assay for riboflavin. In our experiments, L-lyxoflavin was tested for riboflavin activity in both prophylactic and curative assays. The ration of Street(13) was modified so that the daily vitamin supplement contained 20 γ B_1, 20 γ B_6, 200 γ calcium pantothenate, 250 γ p-aminobenzoic acid, 500 γ i-inositol, 2 γ biotin, 2 γ folic acid, 1 mg niacin and 4 mg choline chloride. Male, weanling Sprague-Dawley rats were used in these experiments. In the prophylactic assay 3 levels of L-lyxoflavin (7, 35 and 70 γ per day) and 2 levels of riboflavin (3.5 and 7 γ per day) were employed in addition to a negative control. Ten rats were used in each group. The results are plotted in Fig. 2. The greatest growth-promoting effect of lyxoflavin was manifested within the first 2 weeks of the assay, after which time the growth rate declined in contrast to the sustained weight gain of the rats receiving riboflavin. It is also

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* Roche Publication No. 317.

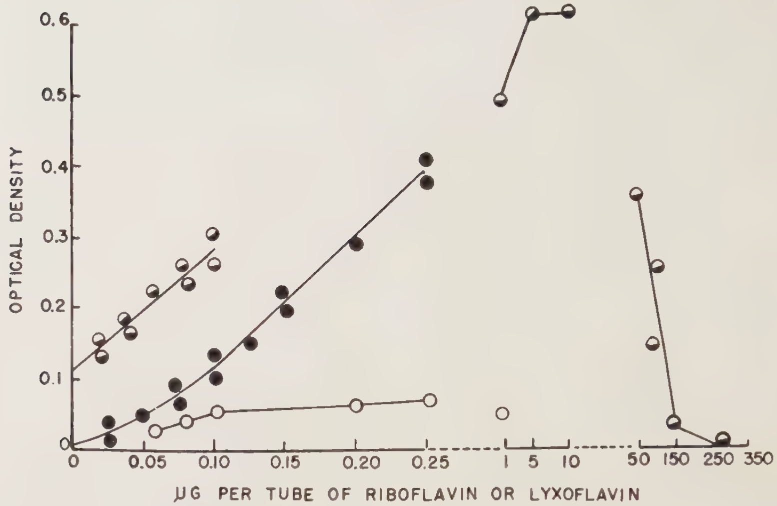

FIG. 1. Growth of *L. casei* with riboflavin and/or L-lyxoflavin in a riboflavin-deficient medium. ●——● riboflavin, ○——○ L-lyxoflavin, (half closed circle)——(half closed circle) riboflavin 0.1 γ + L-lyxoflavin.

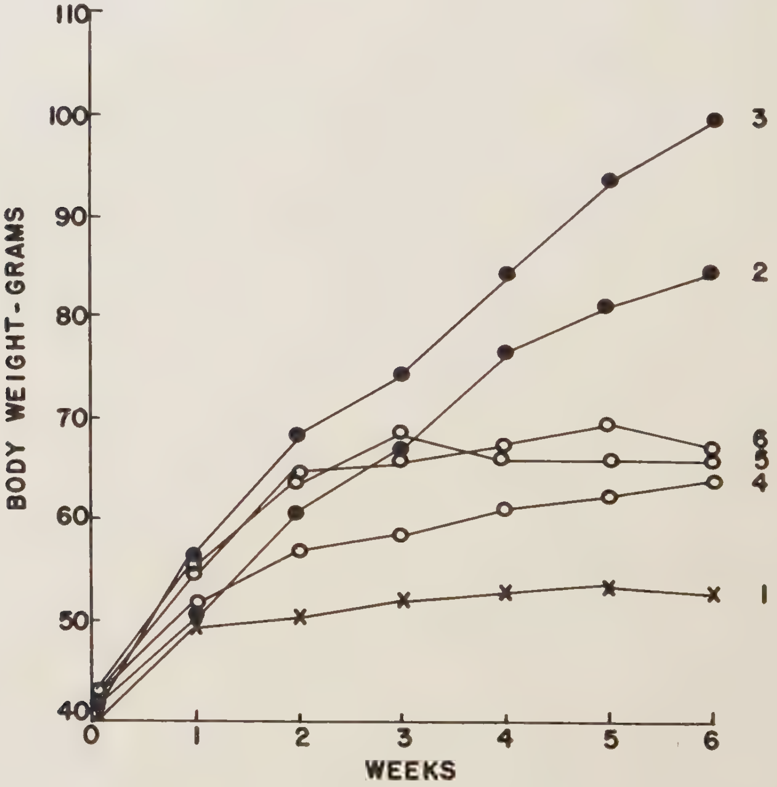

FIG. 2. Growth of riboflavin-deficient rats after dosage with riboflavin and L-lyxoflavin: prophylactic assay.

Group		Dosage in γ per day
×——×	1	none
●——●	2, 3	riboflavin, 3.5 and 7, resp.
○——○	4, 5, 6	L-lyxoflavin, 7, 35 and 70, resp.

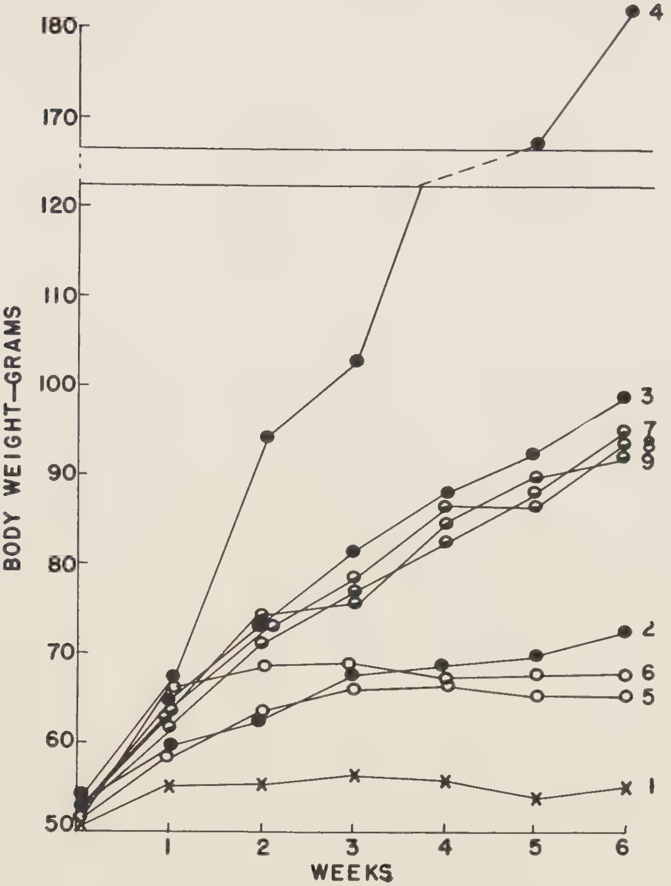

FIG. 3. Growth of riboflavin-deficient rats after dosage with riboflavin and/or L-lyxoflavin curative assay.

	Group	Dosage in γ per day
×——×	1	none
●——●	2, 3, 4	riboflavin, 3.5, 7, and 20, resp.
○——○	5, 6	L-lyxoflavin, 20, and 70, resp.
(Half closed circle) —— (Half closed circle)	7, 8, 9	riboflavin, 3.5 + L-lyxoflavin 3.5, 7, and 70, resp.

of interest to note that there was little difference in the weight gain after 6 weeks among the groups receiving the three levels of lyxoflavin.

In the *curative assay,* the rats were given the depletion diet for 3 weeks at the end of which time they were divided into groups of 10 each according to weight. In these experiments, lyxoflavin was fed a) alone (20 and 70 γ per day) and b) at 3 levels (3.5, 7.0 and 70 γ per day), each combined with 3.5 γ of riboflavin. In addition, 3 levels of riboflavin were included (3.5, 7.0 and 20γ per day). The results are shown in Fig. 3. Again, as in the prophylactic assay, the greatest slope in the growth curve was observed within the first two weeks of the assay for the groups receiving lyxoflavin alone, and little difference in growth rate was noted between the 2 levels of lyxoflavin. Of particular interest is the fact that those groups of rats receiving 3.5 γ of riboflavin plus 3.5 γ of lyxoflavin grew as well throughout the assay period as those receiving 7 γ of riboflavin. However, the groups receiving higher levels of lyxoflavin combined with 3.5 γ of riboflavin did not attain higher weights.

In another experiment it was found that a

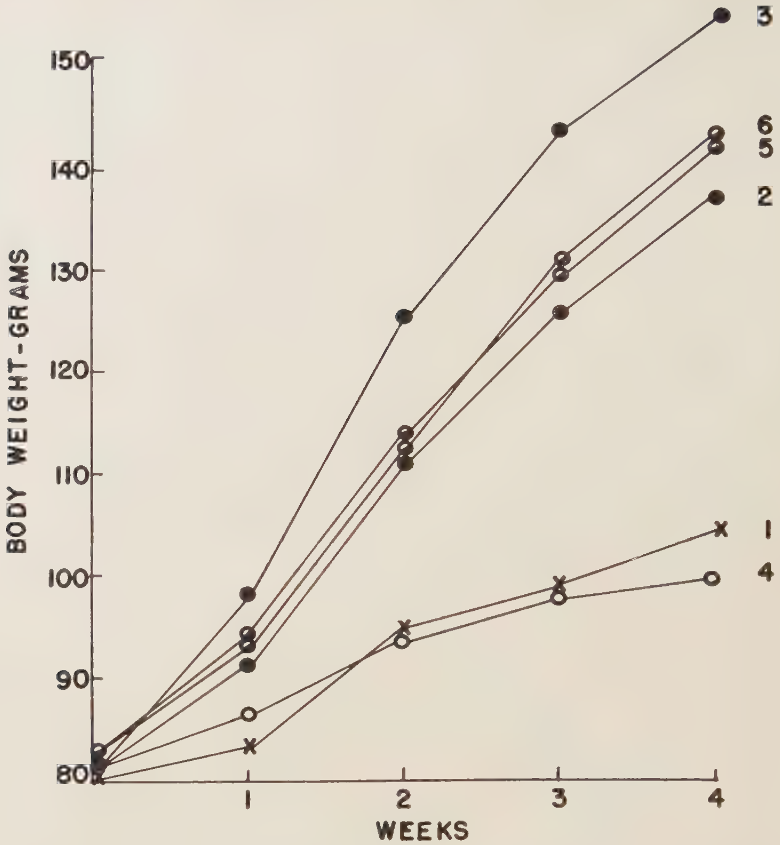

FIG. 4 Growth of pantothenic acid-deficient rats after dosage with pantothenic acid and/or L-lyxoflavin.

	Group	Dosage in γ per day
×———×	1	none
●———●	2, 3	calcium pantothenate, 35 and 70 γ, resp.
○———○	4	L-lyxoflavin, 20 γ
(Half closed circle) ——— (Half closed circle)	5,6	calcium pantothenate, 35 γ + L-lyxoflavin, 7 and 20 γ, resp.

combination of 1.75 γ of lyxoflavin and 1.75 γ of riboflavin produced a similar growth response in a curative assay as 3.5 γ of riboflavin. No further weight gains were observed in groups receiving 2.5 or 10 γ of lyxoflavin in combination with 1.75 γ of riboflavin.

In order to determine whether the growth-promoting activity of lyxoflavin would be exhibited only in the presence of suboptimal levels of riboflavin in the diet, lyxoflavin was fed to rats receiving the same diet but containing optimal amounts of riboflavin and deficient in pantothenic acid. Lyxoflavin was fed alone and in combination with a suboptimal level of calcium pantothenate and compared to groups receiving calcium pantothenate alone (Fig. 4). Under these conditions L-lyxoflavin was inactive in promoting growth in pantothenic acid deficient rats and combinations of L. lyxoflavin and calcium pantothenate were no more effective than that level of calcium pantothenate alone.

Chick experiments. Bruins *et al.*(8) fed lyxoflavin to chicks receiving a ration lacking in unknown factors and found that it produced a small but consistent stimulation in weight at levels of 3 to 30 mg per kilo of ration. There were, however, no data given with comparable levels of riboflavin. Accordingly, we have repeated part of this chick work.

TABLE I. Comparative Effectiveness of L-Lyxoflavin and Riboflavin in Producing Weight Response in the Chick.

Supplement to basal ration, mg/kg	Total gain in 2 wk assay period g	Stand. error of mean
0	77	± 3.6
L-lyxoflavin, 3	72	± 3.8
L-lyxoflavin, 30	82	± 3.5
Riboflavin, 3	85	± 3.8
Riboflavin, 30	81	± 4.2

The ration of Bruins *et al.*(8) was supplemented with 2 levels of lyxoflavin and similarly with 2 levels of riboflavin. One-day-old, single comb, white Leghorn chicks were given the basal ration for 3 days at the end of which time runts and giants were culled and the remaining chicks divided according to weight into 5 groups of 20 chicks each. The chicks were housed in an electrically heated brooder for the 2-week assay period. The results are given in Table I. Small differences were obtained at the higher level of lyxoflavin and at both levels of riboflavin, showing that the small weight gains elicited with high levels of lyxoflavin can be obtained equally well with the same dosage of riboflavin. In any case, the differences from the control group are not statistically significant. However, it is interesting to note that the birds in these 3 groups had superior feathering in comparison to those in the groups receiving the unsupplemented basal or lower level of lyxoflavin which showed scratched and torn skin.

Discussion. From the results of the above experiments it appears that L-lyxoflavin has a limited capacity to replace riboflavin in the nutrition of *Lactobacillus casei* and the rat. Furthermore, for both *L. casei* and the rat, the activity of lyxoflavin appears to vary depending upon the relative levels of riboflavin and lyxoflavin. In this respect, it appears to resemble in activity the d- and l-arabityl analogs of riboflavin in *L. casei*(9) and d-xylityl and l-arabityl analogs in the rat(10,11). The activity of lyxoflavin when fed without riboflavin may vary depending on the stores of riboflavin in the organism or the presence of riboflavin in the diet or medium. Thus, in contrast to the riboflavin activity in the present experiments, lyxoflavin has been reported devoid of activity for the riboflavin deficient rat (7) and for *L. casei* in the absence of riboflavin(8). Recently Shorb(14) has demonstrated that L-lyxoflavin has some riboflavin activity for *Lactobacillus lactis* and confirmed the activity for *L. casei* in the presence of riboflavin.

Wahlstrom and Johnson(15) have described a differential assay procedure for mixtures of riboflavin and lyxoflavin involving the measurement of fluorescence after Florisil purification and microbiological assay with *L. casei.* It is surprising that these authors found no microbiological activity for lyxoflavin in mixtures with riboflavin. This finding is, of course, a necessary prerequisite for the application of the differential assay. Our findings, which have been confirmed by Bruins *et al.*(8) and Shorb(14), that in the presence of riboflavin lyxoflavin has activity for *L. casei* ranging from practically 100% at low levels to inhibition at high levels, would preclude the use of these strains of *L. casei* for this method of assay.

Emerson and Folkers(6,7) obtained a weight response with a daily administration of 150 γ of lyxoflavin in rats fed a soybean meal-dextrose diet containing 0.5% desiccated thyroid equivalent to that obtained when 10% liver or fish meal were added to the diet. Ershoff(16), on the other hand, noted that lyxoflavin was ineffective as an antithyrotoxic factor for the rat. Under his conditions, lyxoflavin failed to elicit a response when fed at levels of 30 and 100 mg per kilo of casein-sucrose diet containing 0.5% desiccated thyroid. At the higher level, the daily dosage of lyxoflavin would be expected to be greater than that administered by Emerson and Folkers(6,7). Liver, however, produced a weight response and prevented the glandular lesions observed in rats fed this thyroid-containing ration. It is apparent that liver counteracted the effect of thyroid powder when fed to rats receiving either a casein-sucrose(16) or soybean-meal dextrose ration(6,7), whereas lyxoflavin was only effective when fed with the soybean-meal dextrose ration(6,7). It is possible that under certain conditions, lyxoflavin may stimulate the synthesis of the liver factor by the intestinal flora. For example, it has been shown(17) that riboflavin can partially replace vit. B_{12} in the nutrition of rats receiving a diet containing thyroxine. Hartman *et al.*(18) also noted that riboflavin can replace vit. B_{12} in the rat and demonstrated that its activity is due to increased synthesis of vit. B_{12} by the intestinal flora. A similar utilization of lyxoflavin, which has been shown to have riboflavin activity under certain conditions, may account for the growth effects observed.

Wahlstrom and Johnson(15) recently reported that lyxoflavin stimulated the growth of baby pigs fed a low fat basal ration containing 0.01% protamone, although the pigs did quite well on the basal ration devoid of any known lyxoflavin. These authors point out that lyxoflavin may exert its effect in a manner similar to that of antibiotics or surfactants rather than as a new member of the vit. B complex. In view of our evidence that riboflavin exerts the same effect as lyxoflavin in promoting growth of chicks on a diet deficient is unknown factors, it is possible that riboflavin would also be active in the rat(6,7) or the pig(15) under conditions where lyxoflavin has been found to stimulate growth. It appears desirable to test riboflavin in any circumstances where lyxoflavin has activity before drawing any conclusions regarding unique functions for lyxoflavin.

Summary. Lyxoflavin has been shown to possess a limited ability to replace riboflavin in the nutrition of *L. casei* and the rat. In chicks, large doses of lyxoflavin produce small weight gains, which, however, can be matched by equal doses of riboflavin. The present evidence does not warrant the classification of lyxoflavin as a new vitamin.

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Effect of Citrovorum Factor upon Tyrosine Metabolism in Clinical Scurvy.* (19777)

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Administration of tyrosine to guinea pigs (1-3), monkeys(4), infants(5,6) or adult men(7) with ascorbic acid deficiency results in the urinary excretion of abnormal quantities of hydroxyphenyl compounds, which decrease

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† Welch Fellow in Internal Medicine of the National Research Council.

TABLE I. Effect of Citrovorum Factor and Ascorbic Acid Upon l-Tyrosine Metabolism in a Scorbutic Man.

Days of study	Period of study	Buffy coat vit. C, mg %	Capillary fragility, 0-4+	Medication daily			Urinary
				l-Tyrosine, G p.o.	citrovorum factor, mg-i.m.	Ascorbic acid, G. p.o.	tyrosyl excretion, G/day
1-5	I	0	4+				.24
6				10	6		2.18
7-10	II		4+				6.45
11-15	III						8.34
16		0	4+			1	3.04
17-20	IV		0				.31
21-25	V	23.9	0				.19

promptly following ascorbic acid therapy. While pteroylglutamic acid (PGA) administered to scorbutic guinea pigs(3), and infants in large doses(6,8) has been reported to correct this hydroxyphenyluria, others have failed to demonstrate an effect of PGA in ascorbic acid-deficient infants(9), monkeys (4) or adult men(10). Recent investigations indicating that ascorbic acid augments the conversion of PGA to the citrovorum factor (CF)(11-14) suggest that CF might be involved in tyrosine metabolism. The present study was undertaken to determine the effect of CF upon the metabolism of tyrosine in an adult scorbutic man.

A 65-year-old bachelor was the subject of this study. On hospital admission he described a grossly inadequate dietary intake during the preceding year and the ingestion of little, if any, of the foods ordinarily providing ascorbic acid. Clinical evidences of scurvy included multiple petechial perifollicular hemorrhages and ecchymoses especially over the lower extremities, but also apparent on the forearms. There was an hyperkeratotic folliculosis over the abdominal wall and the thighs, and the hairs in these areas were brittle and crinkled. The gums were not hypertrophic. Ascorbic acid was not present in the buffy coat of peripheral venous blood. A tourniquet test was markedly positive. Bleeding time, coagulation time, prothrombin time, and platelet count were within normal limits. The hemoglobin was 12.5 g per 100 ml. All of the clinical manifestations of scurvy healed promptly after adequate therapy with ascorbic acid. Immediately upon hospitalization this patient was provided with an ascorbic acid deficient diet consisting of boiled milk, boiled rice, crackers, sugar, coffee, and water, which was continued throughout the study. The 24-hour urine volumes were collected in 10 ml of 1:5 mixture of toluol and glacial acetic acid. Suitable aliquots were stored in a refrigerator until analyzed for hydroxyphenyl compounds ("tyrosyl" derivatives) by the method of Medes(15), a modification of that of Folin and Ciocalteu(16). The tyrosyl derivatives measured by this method include tyrosine, p-hydroxyphenyl lactic acid, and p-hydroxyphenylpyruvic acid. Buffy coat ascorbic acid levels were done by the method of Butler and Cushman(17). An estimate of the number of hemorrhages occurring on a forearm after application of a blood pressure cuff to the upper arm at 100 mm Hg pressure for 10 minutes served as an index of capillary fragility.

Results. The results of this study are presented in Table I. During the initial 5 days of study while the patient ingested the diet described above, the daily urinary tyrosyl excretion ranged from 0.19 to 0.30 g daily. During the second 5-day period 10 g of l-tyrosine‡ was given orally and the tyrosyl excretion ranged from 2.18 to 7.55 g daily and averaged 5.60 g daily. Then 6 mg of CF§ was given intramuscularly daily for 5 days while the l-tyrosine was continued orally. Tyrosyl excretion ranged from 6.24 to 13.20 g daily and averaged 8.34 g daily. Tyrosine was continued during the fourth period of study, CF was discontinued, and 1 g ascorbic acid was given orally daily. Urinary tyrosyl excretion decreased promptly and progressively to values comparable to those observed during the initial study period, and ranged from 3.04 to .08 g daily. Tyrosine was discontinued and ascorbic acid continued during the final period, and tyrosyl values ranged from .11 g to .27 g daily.

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‡ Provided by Merck and Co., Rahway, N. J.

§ "Leucovorin", a synthetic material with citrovorum factor activity, was provided by Lederle Laboratories Division, American Cyanamid, Pearl River, N. Y.

Discussion. The patient with scurvy studied demonstrated an abnormality in tyrosine metabolism which was promptly reversed by ascorbic acid, in confirmation of previous observations in adult man(7). CF administered parenterally did not decrease urinary tyrosyl excretion. Thus, the results obtained in the man with scurvy studied were similar to those observed in the ascorbic acid deficient monkey, in whom a failure of CF to alter the metabolism of administered tyrosine has been noted(18).

A failure of CF to alleviate dietary ascorbic acid deficiency in guinea pigs has been reported(12). CF in the doses given did not alter capillary fragility in the patient reported here, or in an additional scorbutic patient in whom these observations were made before, during and after the intramuscular administration of 1 mg of CF daily for 10 days.

Summary and conclusions. 1. A patient with scurvy excreted large quantities of tyrosyl derivatives in the urine when given l-tyrosine orally during the scorbutic state. The tyrosyl excretion was not decreased by the intramuscular administration of 6 mg of citrovorum factor daily for 5 days, but was promptly corrected by 1 g of ascorbic acid administered orally daily. 2. Citrovorum factor in the doses indicated did not alter the abnormal capillary fragility of scurvy in 2 patients.

The author wishes to thank the Misses Alice Ballou and Virginia Olsson for technical assistance.

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Effects of 2,4-Dinitrophenol upon Oxygen Consumption and Ciliary Activity in the Ctenidia of *Mytilus*.* (19778)

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Nitrated phenols such as 2,4-dinitrophenol (DNP) are known to have the ability to increase the consumption of oxygen and the production of heat by cells and tissues(1). Although the mechanism of action of DNP is obscure, it has been suggested that it "uncouples" the energy-yielding steps of metabolism from those which make use of the released energy(2). Direct observation of energy utilization by cells treated with DNP should contribute to knowledge of its mechanism of action. This report describes measurements of oxygen consumption and ciliary action in molluscan ciliated epithelium treated with DNP. The inhibition of phosphate uptake in this tissue by other chemical agents has been reported previously(3).

Materials and methods. Excised ctenidia (gills) of *Mytilus edulis* L., the edible mussel, were transferred to petri dishes of filtered sea water cooled to 20° or 21°C. The ctenidia were teased apart if necessary and cut into squares or rectangles about 5 mm long.

The DNP was first titrated with NaOH to convert it to its sodium salt. The stock solution of DNP, made in filtered sea water buffered at pH 8.0 with 0.03 M glycine, was diluted further with sea water for use.

Oxygen consumption was measured manometrically at 20°C in 15 ml Warburg flasks. Each flask contained 2 ml of buffered sea water and 3 or 4 ctenidial fragments. Ciliary activity was measured by observing the rate at which pieces of ctenidia moved across the bottom of a petri dish containing sea water or DNP solution(4). Each piece was timed first in sea water and then after immersion in DNP solution. Controls were run in sea water alone. The time required for the tissue fragment to move 0.5 cm was measured 5 times in 5 minutes, beginning 15 minutes after immersion; the observations were then converted to velocities and averaged.

Results and discussion. The results of the experiments are summarized in Table I. In general the data indicate that DNP augments oxygen uptake in excised *Mytilus* gill with a maximal effect at a concentration between 2.9 x 10^{-4} and 1.2 x 10^{-3} M. (Graphic interpolation gave 5 x 10^{-4} M as the most effective concentration of DNP). At the highest concentrations employed there appeared to be a return to normal (or perhaps subnormal) oxygen consumption. Similar maxima have been observed in a variety of animal tissues and microorganisms(1).

DNP had an inhibitory effect on ciliary beat. Inhibition was estimated to be 50% effective when the concentration of DNP was between 2.9 x 10^{-4} M and 5.8 x 10^{-4} M (graphic estimation giving 4 x 10^{-4} M as the concentration for 50% inhibition) and was complete in the presence of 7.7 x 10^{-3} M DNP. Thus the concentration at which the DNP caused the greatest change in ciliary activity coincides closely with the concentration at which it caused maximal augmentation of respiration.

The augmentation of energy release has been visualized by Peiss and Field(1) as the inactivation of a "brake" type of rate-controlling mechanism. Spiegelman and Kamen (5) have shown that in yeast, DNP causes a decrease in the rate of assimilation of phosphorus, probably into energy-rich phosphate bonds. Tyler(6) has suggested that there may be more than one site of action for DNP.

The results reported here suggest that oxidative breakdown of intracellular foods is accelerated by DNP at the same concentration that interrupts ciliary activity. Although our data do not provide additional support for the multiple action of DNP, they do suggest

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TABLE I. Effects of DNP on Oxygen Consumption and Rate of Ciliary Activity of *Mytilus* Gill.

Final conc. DNP, mols · l^{-1}		Oxygen consumption, normal = 1		Ciliary activity, normal = 1	
		n	Mean and std error	n	Mean and std error
1.1	$\times 10^{-6}$	4	.95 ± .03		
2.3		4	.95 ± .01		
4.5		4	.94 ± .02		
9		4	.89 ± .05		
1.8	$\times 10^{-5}$	4	1.08 ± .03	3	.91 ± .02
3.6		4	.97 ± .05	3	.91 ± .06
7.2		5	1.39 ± .02	3	.74 ± .21
1.4	$\times 10^{-4}$	11	1.48 ± .05	3	.68 ± .15
2.9		19	1.75 ± .07	3	.74 ± .02
5.8		23	1.88 ± .02	3	.41 ± .07
1.2	$\times 10^{-3}$	10	1.49 ± .05	3	.16 ± .04
2.3		6	1.35 ± .06	3	.06 ± .03
4.6		6	1.11 ± .02	3	.14 ± .05
7.7				3	No activity

that the augmentation of respiration and the uncoupling of endergonic processes may be closely linked. Further studies of metabolism in ciliated cells may clarify the site of action of DNP.

Summary. 1. The effects of 2,4-dinitrophenol (DNP) upon oxygen consumption and ciliary activity in the excised ctenidia of *Mytilus edulis* were investigated. 2. The maximal stimulation of oxygen uptake was estimated to occur when the DNP was 5 x 10^{-4} M. 3. Ciliary beat was increasingly inhibited as the concentration of DNP was raised. 50% inhibition was estimated at 4 x 10^{-4} M DNP, and inhibition was complete in the presence of 7.7 x 10^{-3} M DNP. 4. The possible role of DNP in uncoupling exergonic from endergonic processes receives support from the close coincidence of these effective concentrations. The close correspondence also suggests a single site of action for DNP.

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Chemotherapy of Experimental Tuberculosis with Quinoline Derivatives.

(19779)

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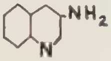
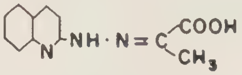
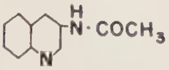
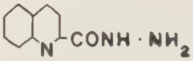
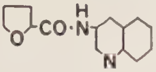
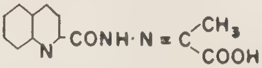
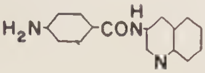
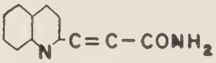
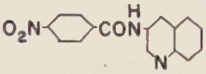
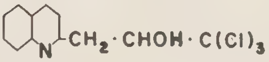
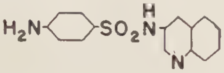
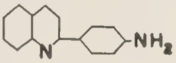
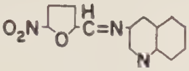
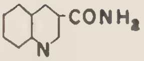
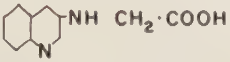
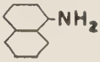
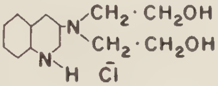
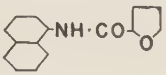
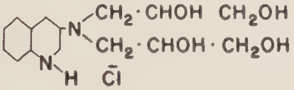
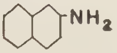
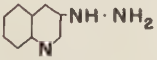
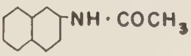
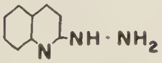
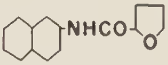
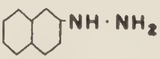
New derivatives of aminoquinolines were investigated for tuberculostatic action and for therapeutic effect on tuberculosis in guinea pigs. Derivatives of 3-aminoquinoline were emphasized, as this compound is highly bacteriostatic. In the present experiments a number of allied compounds were also tested, including hydrazine derivatives of quinoline and naphthylene as well as some 2-substituted quinolines. A few previously reported compounds were included for comparison.

Methods. The bacteriostatic studies were performed on virulent tubercle bacilli, strain No. H37Rv in Kirshner's synthetic asparagin

TABLE I. Tuberculostatic Action on Strain H37Rv.*

No.	Compound	Min. Inhib. Conc. mg. %	No.	Compound	Min. Inhib. Conc. mg. %
1		0.05	13		5.
2		5.	14		5.
3		0.05	15		5.
4		1.25	16		10.
5		5.	17		>5.
6		0.5	18		1.
7		0.05	19		5.
8		0.5	20		0.5
9		10	21		2.5
10		0.5	22		0.05
11		>5.	23		0.05
12		1.25	24		0.05
			25		0.5

* Above compounds were prepared in this laboratory excepting No. 1, 12, 20, 22, and 25, which were obtained from Eastman Kodak Co. (highest purity).

and glycerine media at a pH of 7.2. The chemicals were dissolved with a minimum of heating in acetone or in 0.8% sodium hydroxide to make 0.5% solutions. Subsequent dilutions were made directly into the media without further heating. Each flask was inoculated with one loopful (4 mm in diameter) of a 14-day-old surface culture of tubercle bacilli. The surface cultures were read at 13 days, using the highest dilution in which there was less than 50% of control growth as an end point.

Animal experiments. Twelve guinea pigs weighing an average of 400 g were used to evaluate each compound. The animals were inoculated in the left groin with 0.1 mg wet weight of H37Rv tubercle bacilli. The drugs were administered daily, orally by pipette, in

TABLE II. Summary of *In Vivo* Experimental Data.

No.	Compound	Avg daily dosage, mg/animal	Path. ratio*	Wt change deviation from control, g	Hemoblob. deviation from control, %
4.	N-(3-quinolyl) *p*-aminobenzamide	200	1.4	—26	+6
5.	*p*-nitrobenzamide	80	1.1	+60	0
6.	*p*-aminobenzene sulfonamide	130	1.7	—46	0
3.	furoylamide	50	.81	—22	+6
10.	N,N-dipropyleneglycol-3-aminoquinoline	100	.87	—45	+5
14.	Quinaldinic acid hydrazide	10	1	—27	—12
15.	Pyruvic acid hydrazone of #14	12	.27	—38	+2
26.	*p*-aminosalicylic acid (sodium salt)	250	2	+30	0
27.	Streptomycin	†	4	+58	0

* Pathological ratio is the quotient of the average numerical pathological rating of control animals divided by that of treated animals.

† Streptomycin given by hypo 40 mg 3 times weekly. Both streptomycin and *p*-aminosalicylic acid were included for comparison.

aqueous suspension. Therapy was started on the day of infection. The dosage of all drugs was increased gradually for the first 2 weeks to the approximate chronic maximum tolerated dose. The experiments were terminated at the end of 7 weeks. Pathology was evaluated numerically, based on a maximum of 4 for each organ (glands, spleen, liver, and lungs). Hemoglobin and weight determinations were made at the end of the experiment.

Results and discussion. The *in vitro* bacteriostatic results are recorded in Table I. Derivatives of 3-aminoquinoline (Nos. 1-11) inhibited growth in varying degrees. The parent compound (No. 1) and its furoyl (No. 3) and 5-nitrofuranil (No. 7) derivatives were all highly bacteriostatic. This may be due to the ease of hydrolysis of the derivatives. Quinoline-3-hydrazine (No. 11), wherein there is a substitution of a hydrazine for the amino group of 3-aminoquinoline, negated the inhibiting effect of the latter compound. Quinoline-2-hydrazine was slightly more bacteriostatic than the 3 isomer, but its corresponding pyruvic acid hydrazone (No. 13) was less active. The hydrazide of quinaldinic acid (No. 14) was a poor inhibitor. The naphthylamine derivatives (Nos. 20-25) were included mainly for comparison, and in general these compounds were more consistently bacteriostatic than the corresponding quinoline analogues. The most active derivatives of 3-aminoquinoline and β-naphthylamine were those most easily hydrolyzed. There was no correlation between activity and solubility, Ka values, or partition coefficients.

The *in vivo* results are summarized in Table II. These compounds were not chosen because of high bacteriostatic activity but rather for their diverse physico-chemical properties such as solubilities, degree of basicity, and for their structural relationship to known biologically active compounds. Of the 7 new compounds evaluated, one showed some therapeutic effect, namely the sulfonamide derivative (No. 6). This compound was toxic as evidenced by weight loss of the animals. The therapeutic results compared unfavorably with those of streptomycin and p-aminosalicylic acid. The sulfonamide derivative described above has been prepared by others(1) but has not been evaluated on many organisms, possibly because of its relatively high dissociation constant.

Summary. 1. Nineteen quinoline derivatives, including eleven 3-aminoquinoline derivatives, were evaluated for tuberculostatic action upon *M. tuberculosis* H37Rv. 2. Seven quinoline derivatives were tested *in vivo* in experimentally induced tuberculosis in guinea pigs. 3. The relationship between chemical structure and tuberculostatic action was discussed.

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The technical assistance of Miss Elizabeth Girdish is acknowledged.

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Relation between Serum Complement and Plasma Electrophoretic Fraction Levels in Human Tuberculosis.* (19780)

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Complement is an important constituent of blood plasma and other body fluids as evidenced by its intimate association with antigen-antibody complexes, its participation in bacteriolysis, hemolysis, complement fixation, phagocytosis and other immunological reactions. It is a complex of plasma proteins associated for the most part with the globulins. As it occurs in serum as less than 1% of the total protein (0.03 to 0.05 mg C′ N per ml serum)(1), (0.15 to 0.20 mg C′ per ml serum)(2), electrophoretic measurements are not possible(3). Its marked activity in lysing sensitized erythrocytes, however, allows detection and measurement in small quantities.

Stavitsky, Stavitsky, and Ecker(6) have demonstrated marked and prompt reduction in complement after reinjection of a variety of antigens into previously immunized rabbits and give evidence that *in vivo* binding of complement by antigen-antibody complexes is responsible. Ecker and his associates(4) have summarized the numerous studies directed toward attempting to relate complement actvity to various infectious diseases and stages of disease as well as to serum sickness. In tuberculosis conflicting reports have appeared in the literature. Objectives of the present study were to determine 1) whether serum complement levels in human pulmonary tuberculosis differed from levels in normal, apparently healthy humans, 2) the effect of the administration of ACTH on complement levels in human pulmonary tuberculosis and 3) correlation of hemolytic complement activity with electrophoretic levels of plasma proteins in a group of patients receiving ACTH.

Materials and methods. Complement titration. Complement titrations were performed on 3 groups of human subjects. Twenty normal, apparently healthy individuals, both tuberculin-positive and tuberculin-negative, on whom a total of 54 titrations were made, served as a control group. The second group consisted of 37 patients in various stages of pulmonary tuberculosis on whom a total of 59 titrations were performed. The third group consisted of 10 tuberculous patients who were to receive ACTH on whom a total of 65 titrations were done. Complement levels on most of these patients were obtained before ACTH was initiated and at intervals during several months of treatment.

Titrations were performed promptly on freshly drawn serum using the method of 50% hemolysis. Sheep blood was collected in Alsever's solution(7). The technic of Wadsworth, Maltaner, and Maltaner(7) was modified by the substitution of readings on the Coleman Universal spectrophotometer for visual color standards. Erythrocyte suspensions were standardized spectrophotometrically to correspond to 1,000,000 cells per ml. Hemolysin was used at its optimal concentration(7). Mg^{++} and Ca^{++} were not employed in the diluting fluid; it was felt that comparative figures of group analyses of the human sera of this study nevertheless have significance, inasmuch as optimal amounts of these ions raise 50% titers of guinea pig sera by a rather constant amount (78% to 88%), and of human serum by about 28%(19).

The method of calculating the 50% unit was based on the equation of von Krogh(8):

$$\text{Log } x = \log K - 1/n \log y/1 - y$$

where x is the amount of complement and y is the resulting degree of hemolysis. 1/n represents the slope of the line. When $y = 0.5$ (50% hemolysis), $y/1 - y = 1$, $\log x = \log K$ and $x = K$. Accordingly amounts of complement (x) were plotted against $y/1 - y$ on

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* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

TABLE I. Statistical Analysis of Complement Levels.

Group	Source of variation	D.F.	Variance	F	Required F 5%	1%
Normal subjects	Between patient	19	77.16	4.39*	1.90	2.50
	Within "	34	17.54			
Tuberculous patients	Between patient	36	52.75	1.79	1.95	2.62
	Within "	22	29.36			
Tuberculous ACTH patients	Between patient	9	74.77	3.10*	2.05	2.75
	Within "	35	24.08			

* F value highly significant.

TABLE II. Comparison of Mean 50% Units.

Group	Mean 50% unit in ml
Normal subjects	.0030
Tuberculous patients	.0026
Tuberculous ACTH patients	.0025

logarithmic paper and a straight line fitted through the points. The 50% unit is the ordinate of the point of intersection of the fitted line and the line $y/1 - y = 1$ where the degree of hemolysis is 0.5 (50%). The logarithmic method is advantageous in that it allows observed values which do not fall on the straight line to be discarded. Obviously inconsistent readings would therefore not erroneously influence the value of the 50% unit.

Methods of analysis. Evaluation of the results of the 50% hemolytic units were obtained by analysis of variance and correlation and regression technics which provide objective tests of the significance of observed differences(9,10). The analysis of variance is advantageous in that variation within each group is measured as well as variation between groups and estimates of variance are computed independently from these 2 sources.

Electrophoretic fractionation. Diagrams were obtained under very nearly the same experimental conditions in order to make comparisons possible. The plasma from fasting heparinized blood samples was diluted 1-6 with barbital buffer solution, pH 8.6 at 25°C, ionic strength 0.1; electrophoresis was carried out in the tall form of the standard 11 ml Tiselius cell in a water bath at 1°C. Ten milliamperes of current were passed for at least 3 hours. Areas of the peaks were obtained from enlarged tracings of the anode pattern photographs. Percent of total plasma proteins was determined according to the procedure of Tiselius and Kabat(11). Quantities as grams percent represent products of the percentages and the total proteins determined by the conventional Kjeldahl method, using a nitrogen factor of 6.25.

Results. Group comparison of complement levels of normal subjects, tuberculous patients and tuberculous patients receiving ACTH. To arrive at a basic measure of variation, 2 sources were considered; variation in complement levels from patient to patient (between patients), and fluctuation among repeated tests on individual patients (within patients). The variance from these 2 sources was separated and examined independently before being used as the basis of tests of significance of differences among average complement levels for the 3 test groups. The analysis of variance for each of the 3 groups is shown in Table I.

Variation in complement activity is seen to be greater from patient to patient than among repeated tests on individual patients. Conse-

TABLE III. Combined Analysis of Variance.

Source of variation	D.F.	Variance	F	Required F at 5%	1%
Total	177				
Normal vs tuberculous	1	816.83	35.23*	3.93	6.88
Tuberculous vs ACTH tuberculous	1	57.85	2.50	3.93	6.88
Between patients	64	63.08	2.73*	1.45	1.67
Within "	111	23.13			

* F value highly significant.

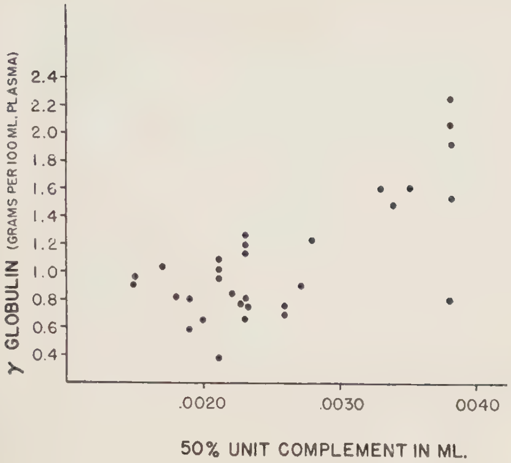

FIG. 1.

quently these 3 groups of data were combined to form an estimate of error against which differences in complement levels among the 3 groups could be tested for significance.

Means of the 3 groups of subjects are shown in Table II and the combined analysis of variance in Table III.

Table II shows that the highest complement activity occurred in tuberculous patients and the lowest in normal individuals (the larger the 50% unit, the less the complement activity). Analysis of variance shows this difference to be highly significant. The variance for the difference between the 2 groups of tuberculous patients, however, is not significant, being of about the same order as the variance between tuberculous patients, indicating that the difference between the tuberculous and tuberculous-hormone groups might be attributed to random sampling.

Comparison of complement levels on individual patients during course of ACTH administration. By plotting complement levels against time it was found that the trend was toward increased activity after long continued treatment. It is not marked nor is it immediate, a noticeable plateau being evident. A significant correlation of 0.32 was obtained between pairs of complement levels and time, indicating increased complement activity with continued ACTH administration beyond any variation attributable to random sampling.

Correlation of complement levels with electrophoretic components. Scatter diagrams prepared by plotting each electrophoretic component (albumin, alpha 1 globulin, alpha 2 globulin, β-globulin, gamma globulin and fibrinogen) against complement levels of identical blood samples showed that only in the case of gamma globulin was a trend evident. Fig. 1 illustrates the tendency for decreased gamma globulin to be associated with increased complement activity. The above sets of a complement-gamma globulin level give a highly significant coefficient of correlation (0.729, calculated F 34.32**, tabulated F at 1% level 7.56).

Discussion. Ecker(4) has pointed out that many apparent contradictions and inconsistencies in the literature can be attributed to lack of uniform technic and to inadequate definition of the limits of normal and abnormal complement titers. In this study an average 50% unit of 0.0030 ml was obtained for the normal group, which closely agrees with that found by Traub (0.0031-0.0032 ml)(12), who found no significant differences attributable to age, sex, blood groups or seasonal influence. Dulaney(5) found the median 50% unit in a group of normals to be 0.0045 ml, while Pohl and Rutstein(13) found a median 50% unit of 0.0049 ml. In this study, on the basis of group comparisons, the complement level of the tuberculous group was significantly higher than that of the normal group. References to complement levels of tuberculous patients expressed in 50% units were not found in the literature.

True complement titers of sera might conceivably be masked by the occurrence of natural heterophile antibody, known to influence the extent of hemolysis. Traub, however, found the 50% complement units of 10 sera which contained large amounts of heterophile antibody to be unaffected(12). Moreover in the present series, heterophile antibody was detected in only a few cases; in any case, dilutions of serum used for complement determinations would have obviated possible error from this source.

ACTH administration is known to result in alterations in serum proteins and gamma globulins may be significantly reduced in a variety of diseases. Some investigators have observed a lag period after ACTH before the decrease in gamma globulin occurs(14). In the tuberculous group in the present study, elevated gamma globulin fractions tended to approach the normal after initiation of treatment. This decrease, coupled with the marked tendency for the association of decreased gamma globulin and increased complement activity, indirectly indicates increased complement activity after the hormone, as does the significant correlation between complement activity and time after treatment. Failure to detect a significant difference in the large group analyses between the means of the tuberculous group and the tuberculous-hormone group might be attributed to inclusion of results during a lag period in some of the treated patients, which would minimize the difference between the means of the 2 tuberculous groups and thus account for lack of agreement in the 2 methods of analysis.

It is known that serum globulin solutions are often anticomplementary(15,16). Davis and his coworkers(16) found that gamma globulin separated from human serum by electrophoresis was anticomplementary in high dilution but that recombination with other constituents, notably β-globulin, inhibited this property. Vaughan, Bayles, and Favour found decreased gamma globulin levels and raised complement titers in 4 cases of lupus erythematosus disseminatus treated with ACTH(17). Carey, Harvey, and Howard (18) confirmed these results in lupus erythematosus and also in periarteritis nodosa treated with ACTH. In the present study, also, a highly significant negative correlation between complement and gamma globulin levels was observed following ACTH treatment. There was complete lack of correlation, however, between complement content and β-globulin, as well as alpha 1 and 2 globulins, albumin and fibrinogen.

If complement activity were mediated through the gamma globulin content, then, in view of the consistent decrease in gamma globulin after ACTH administration in most of the patients in the present study, simultaneous elevation in complement levels would be expected. Results on the hormone group alone show an elevation, indicated by the significant negative correlation between complement levels and time. In the group comparisons, diminished complement activity was not noted in the untreated tuberculous group, which would be expected to have elevated gamma globulins. Unfortunately, electrophoretic patterns were not obtained on this particular group of subjects.

It would appear that a number of immunologically important factors are operative in influencing the amount of titratable complement, such as the binding of complement by "normal" gamma globulin, inhibition of the anticomplementary effect of gamma globulin by β-globulin and albumin, the observed reciprocal relationship of complement and gamma globulin levels in some diseases, the observed *in vivo* binding of complement by antigen-antibody complexes and probably others. Evaluation of results would depend upon knowledge of the interplay of these factors and the extent to which each is operative in any one individual.

Summary. 1. Fifty per cent hemolytic units of complement determined on groups of normal individuals, tuberculous patients and tuberculous patients receiving ACTH showed highest activity in the tuberculous group and least activity in the normal group. Analysis of variance showed differences between the complement activity of normal and tuberculous individuals significant. 2. A significant correlation was observed between complement levels and time after hormone administration indicating a trend toward increased complement activity (decreased 50% unit) after long continued ACTH administration. 3. A highly significant correlation was seen between the concentration of gamma globulin and complement level, increased complement activity being associated with decreased gamma globulin concentration. No correlation between complement activity and other electrophoretic components (albumin, alpha 1 and alpha 2 globulins, β globulin and fibrinogen) was observed.

An expression of appreciation is due Dr. Marjorie Sutherland for the statistical analysis.

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Effect of Adrenal Hormone Overdosage on Bacterial Removal by the Splanchnic Viscera.* (19781)

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An adverse effect of adrenal hormone overdosage on the course of experimental infections has been reported(1,2). A higher incidence of positive blood cultures was noted in animals receiving cortisone who were infected with Group A streptococci and pneumococci(1,3). Some studies have shown a diminution in the inflammatory response(1,4,6). The cause of the increased incidence of bacteremia has not been shown but may be attributed to the failure of the inflammatory response to localize the pathogen at the site of injection or to the failure of the splanchnic vascular system to remove the bacteria after they have entered the blood stream. Gordon and Katsh(5) have demonstrated that adrenal insufficiency lowers endothelial phagocytosis and decreases the removal of particles in the liver and spleen. This report deals with the effects of overdosage of ACTH and cortisone on the ability of an animal to remove a non-encapsulated organism. There is no difference in the removal rate of such an organism before and after chronic adrenal stimulation.

Healthy 4 to 4.5 kg rabbits were maintained on a mixture of rabbit chow and oats with no periods of fasting. The animals receiving adrenocorticotrophic hormone (ACTH, Wilson's) were given 10 units every 4 hours for 24 hours before study. The animals receiving cortisone were divided into 4 groups. Two groups were studied at 4-5 hours after a single intramuscular or oral dose of 25 mg cortisone. The 2 remaining groups were studied after 4 days of therapy with 25 mg of cortisone a day given intramuscularly or orally, the last dose being given 4-5 hours before study. A bacteremia was maintained by constant infusion into a marginal ear vein of hemolytic *Micrococcus pyogenes var aureus*, coagulase positive. The splanchnic removal rate was determined by passing a catheter into the hepatic vein and comparing the number of bacteria found in cultures of the blood drawn simul-

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† Markle Foundation Scholar.

TABLE I. Effect of Adrenal Hormone Overdosage on Removal of Bacteria from Blood Stream.

Treatment	Duration	% removal	No. of observations, animals	P
Control	4 hr	61.5 ± 20 *	19 – 4	
Cortisone acute, oral	4 "	71.7 ± 13.6	24 – 3	<.05
" " parenteral	4 "	73.9 ± 16.4	23 – 4	<.05
" oral	4 days	66.6 ± 20	24 – 5	>.05
" parenteral	4 "	57.67 ± 25.1	36 – 5	>.05
ACTH	24 hr	54.6 ± 26.4	20 – 5	>.05

* Stand. dev.

taneously from the hepatic vein and the heart or inferior vena cava(7). The removal rate is expressed as the per cent of bacteria removed when arterial and venous concentrations are compared. One per cent metycaine was used for local anesthesia.

Results. Table I presents a comparison of the removal rate under the circumstances outlined. The values are expressed as per cent removal with the standard deviation. There is no significant difference between the removal of *Micrococcus aureus* in the control group of animals and in the animals receiving ACTH or cortisone for periods greater than 4 hours. There is a questionable increase in the removal rate when measured 4 hours after administration of a single dose of cortisone.

Discussion. Chronic overdosage with ACTH and cortisone neither increased nor decreased the splanchnic removal of bacteria. These normal rates of bacterial removal suggest that the frequency of blood stream involvement noted by previous experimentation(1,2) does not depend on the failure of removal of organism by splanchnic vascular system. They indicate that the cause of the bacteremia may lie outside the splanchnic removal system and may be attributed to the absence of an inflammatory reaction in the tissues with the result that an infection is not localized and organisms may continually spread into the blood stream. The inflammatory reaction both in the interstitial tissue and in the blood vessels(8) may be of importance in preventing dissemination of organisms. Present studies in progress indicate an alteration in metabolism and function of the cells in the exudate exposed to adrenal hormone.

If the above reasoning is correct, the basic cause of the increased incidence of bacteremia after chronic ACTH and cortisone overdosage is similar to that responsible for the increased incidence of bacteremia in marked leucopenia where removal rates are also normal(9). In the case of ACTH and cortisone, the cells are absent from the lesion because the inflammatory reaction is suppressed; in the case of the animals without white cells, the cells are not available to migrate into the lesion. In both instances, organisms move freely from the local lesions into the blood stream.

The increase on acute administration of cortisone may be a reflection of the heightened phagocytic activity previously described by Dougherty and co-workers(10). The difference between acute and chronic administration calls attention to variations which may arise under these circumstances.

Summary. Chronic administration of adrenocorticotrophic hormone and cortisone had no effect on the removal of *M. aureus var pyogenes* by the splanchnic vascular system. It is suggested that the increased incidence of bacteremia seen after ACTH and cortisone as well as that seen in the leucopenic states is the result of poor localization of the organisms in the tissues. In the former, the reduction in the inflammatory reaction and the change in the metabolism of leucocytes are important. In the latter, white blood cells are not present to migrate into the lesion. There was a questionable increase in removal rate after acute administration (4 hours).

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Counts of Virus Particles by Sedimentation on Agar and Electron Micrography.* (19782)

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The solution of many problems in virology is dependent on the quantitative correlation of number of virus particles or unit virus mass with the biological attributes of the agent. Estimation of mass or particle number in preparations of purified viruses of uniform shape and size can be made simply and fairly accurately by chemical analysis. In other cases, only direct counts of the particles offer assurance of accuracy. One excellent method for this purpose involves the comparison of a known number of latex particles mixed with virus particles in sprayed droplets(1). In another procedure(2), virus is sedimented on a collodion membrane, and counts are made of the particles in a measured area. Success with the first of these methods, however, is dependent on high concentration of virus and, with both, on the absence of interfering extraneous material such as protein or salt.

Recently, a modification of the sedimentation procedure has been developed which retains the high sensitivity demonstrated in the earlier work and, in addition, gives promise of high accuracy with preparations containing protein, salts, and other material which hinder electron micrography or may have a harmful effect on the virus particles when dried with them. This method consists in the sedimentation of the virus onto an agar surface from which it can be removed by means of a collodion pseudoreplica.† In this process, excess protein can be washed away, and the salt remaining diffuses into the agar. It is evident, however, that the applicability of this process, as well as others involving electron micrography, is entirely dependent on criteria for unequivocal identification of the virus particles because of characteristic morphology or a specific means for relating virus activity to the particles counted. Preliminary studies on the use of agar for examination of the virus particles of erythromyeloblastic leucosis have been described(3). In the present paper are given results obtained with the agar technic applied to the enumeration of influenza virus particles in purified preparations and in a comparison of these findings with those observed with other methods of counting.

Materials and methods. Counts were made by sedimentation of the influenza virus on an agar surface; sedimentation of the particles on a collodion membrane; and by the spray technic. The mechanical equipment employed in the sedimentation experiments consisted of an air-driven ultracentrifuge, ordinarily used for analytical work, the rotor of which was fitted with cells specially made for the study.

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† The pseudoreplica is a film which, when stripped off, brings with it the virus particles.

These cells, described previously(2), have a wedge-shaped cavity 1 cm in radial depth and 1 cm^2 at the bottom. A group of 4 cells of similar size and weight were operated simultaneously. Sedimentation was effected by spinning the rotor at 16,300 X g for 30 minutes.

Agar in 1 to 3% concentration in water or buffer solution, as described later, was poured into petri dishes to a depth of 2 to 3 mm and allowed to harden. Pieces 1 cm square were cut out and, with care to avoid damage to the surface, placed on the bottom of the cells. The cells were then filled with the virus preparation, sealed and spun. At the end of the run the fluid was carefully pipetted off and, if necessary, the agar surface was flooded gently with Ringer's solution several times to remove the supernatant fluid. The agar was taken from the cell and placed on a glass slide for convenience in handling. When the excess fluid had evaporated or diffused into the agar, a small glass dish or capsule carrying small drops of 2% osmic acid on the inner surface was inverted over the agar for about 20 minutes. A solution of 0.5% collodion in amyl acetate was then pipetted over the agar surface and drained off by tilting the slide against a piece of filter paper. The collodion film, after drying for 3 to 5 minutes, was torn with a needle about the edges of the agar block and floated off on a water surface by submerging the slide held obliquely as it passed into the water. Since the virus was on the underside, the film was inverted as it was removed from the water in preparation for shadow casting.

Agar was buffered with sodium acetate-acetic acid in the range of pH 4-6; with phosphate at pH 6 to 8; and with sodium borate-boric acid between pH 8 and 9. Stock solutions of the acetate and phosphate buffers of 1.0 M concentration were diluted 1 part with 4 parts of 0.85% NaCl solution containing 0.4% formaldehyde. The borate solution was of 0.5 M concentration and was diluted appropriately to yield the same final buffer salt concentration as the others in the agar. The melted agar in 3% concentration in distilled water was mixed with an equal volume of this solution and poured into petri plates to harden.

The procedures employed for sedimenting the particles on collodion fixed on glass were those previously described(2). Particles were counted by the spray technic as described by Backus and Williams(1). Polystyrene particles mixed with virus were a part of the original sample of Dow latex 580 G for which size and density measurements have been reported(4), and particle number was estimated by weighing samples of the suspension. Bovine serum albumin (Armour) in 0.3% concentration was added to the mixture to outline the drops.

Influenza virus A (PR8 strain) was purified as previously described(5), suspended in Ringer's solution of pH 7.4 and preserved with 0.4% formaldehyde at 0-4°C. All subsequent adjustments of pH of this virus suspension were made with NaOH or HCl and checked with a pH meter with a glass electrode. Measurements of pH were frequently made on the suspensions from which virus was spun, and no significant drift was observed.

Results. Influence of pH. The initial experiments were made to determine the conditions optimum for the sedimentation of the influenza virus on an agar surface and for the quantitative incorporation of the particles in a collodion membrane suitable for electron micrography. In these studies the purified virus in Ringer's solution of pH 7.4 was spun down on the surface of agar prepared in distilled water without the adjustment of pH. It was soon evident that the number of particles observed varied over a considerable range. One factor investigated for possible contribution to these inconsistencies was the pH of the suspension from which the virus was sedimented.

The results of a series of counts through the range of pH 5 to 9 are shown in Fig. 1. The stock suspension containing 4.23 mg of influenza virus per ml was diluted 10,000-fold with Ringer's solution in 5 ml volumes and the pH adjusted to the required value. Two screens were prepared from each agar block and shadow-cast with chromium in the usual way. In Fig. 1, each point is the average of 5 fields in the electron microscope at a magnification of 3,610 X and 25 cm^2 on the negative. All areas in these and the other experi-

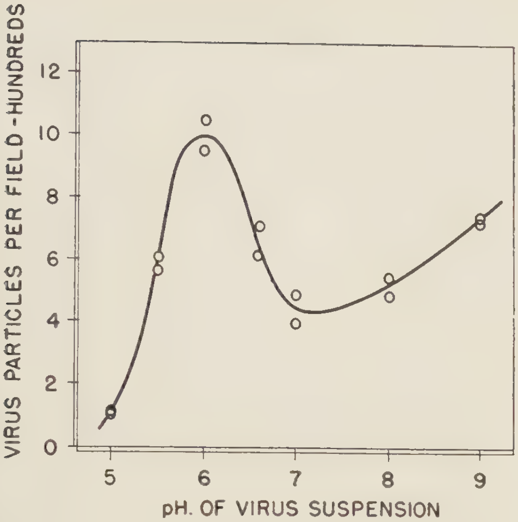

FIG. 1. Relation of pH of influenza virus suspension to number of virus particles sedimented on the agar surface and removed in the pseudoreplica for electron micrography.

ments were chosen by turning the stage controls of the microscope at random, after focusing, and taking the picture where the stage stopped.

A strong dependence of the number of particles on the pH of the virus suspension is clearly evident. Most conspicuous is the maximum at pH 6. At pH 5 and pH 9 there were signs of alteration of the virus particles. The virus was severely aggregated at pH 5. At pH 9 there was no serious aggregation, but the particles in the pictures seemed greatly flattened.

Many of the studies reported here were made by this technic. Later, it was learned that the method could be simplified, and the routine consistency of the results could be improved by adjustment of the pH of the agar rather than that of the suspension of virus particles. Unbuffered saline suspensions of virus quickly take on the pH of the agar block when placed in the cell for sedimentation. An additional refinement of the procedure consisted in the preparation of the agar plates at least a day in advance of the study. Under these conditions, the pH of the agar in the range of 4 to 8 was of little influence on the counts, as seen from the data of the following experiment. A sample of the stock virus suspension was diluted 10,000-fold with 0.85% NaCl solution containing 0.4% formaldehyde with a resulting pH of 5. Portions of this suspension were then sedimented onto agar buffered at various pH values from 4 to 9.

In Fig. 2 each point is the average of the counts of 5 fields chosen at random from a screen of the various preparations indicated. It is evident that the values from pH 5 to 8 were essentially identical; much lower counts were obtained at pH 9, and at pH 4 the values were somewhat erratic. The number of par-

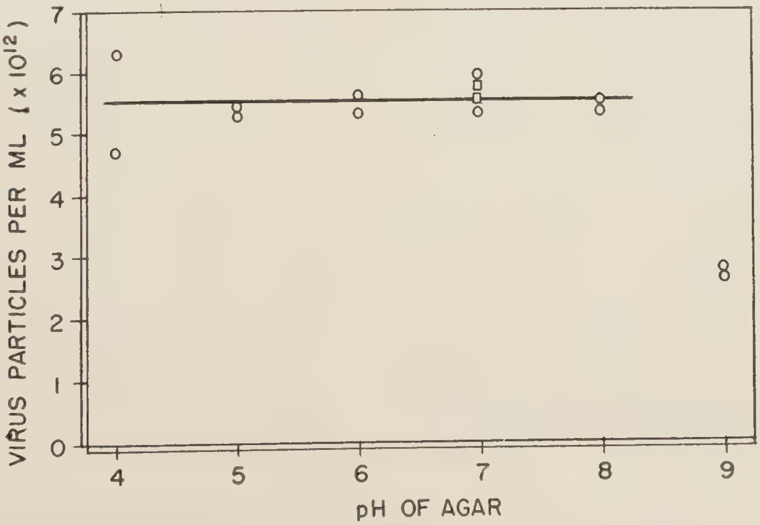

FIG. 2. Relation of pH of agar block to number of virus particles sedimented on the agar surface and removed in the pseudoreplica for electron micrography.

TABLE I. Virus Particle Counts on 25 Cm² Negatives of Purified Influenza A (PR8 Strain) Diluted with Ringer's Solution of pH 6 and Sedimented Directly onto an Agar Surface.

		Micrograph field					
Run	Cell	A	B	C	D	E	
1	1	718	774	630	686	668	
	1	612	650	776	608	816	
	2	794	750	862	874	856	Mean 728
	2	770	798	798	764	692	$\sigma = \pm 13.7\%$
	3	920	774	882	702	836	$\epsilon = \pm\ 2.2\%$
	3	698	668	798	820	677	
	4	626	636	620	818	480	
	4	512	744	688	662	660	
2	1	626	766	704	682	716	
	1	790	656	698	736	650	
	2	894	598	896	744	756	Mean 748
	2	954	892	958	756	922	$\sigma = \pm 16.6\%$
	3	508	758	550	534	558	$\epsilon = \pm\ 3.6\%$
	3	750	560	844	838	734	
	4	952	784	822	874	858	
	4	732	810	570	704	756	

TABLE II. Comparison of Particle Counts of Dow 580G Latex and of Purified Influenza Virus A (PR8 Strain) Obtained by the Various Methods.

Material	Method	Particle No.
Latex	Weighing	6.2×10^{10}
	Sedimentation on collodion	6.1×10^{10}
Virus	Spray	5.2×10^{12}
	Sedimentation on collodion	5.1×10^{12}
	" " agar	5.5×10^{12}
	Nitrogen content	8.9×10^{12}

ticles per ml of the stock suspension, calculated as the average of the data of Fig. 2 from pH 4 to 8, was 5.5×10^{12}. This is not significantly different from the data of the following studies.

Precision of the results. The variations observed in repeated examinations of a given preparation are shown in Table I. For this experiment, the stock virus preparation was diluted 1 to 10,000, as before, with Ringer's solution adjusted to pH 6. Two sedimentation runs were made at 16,300 X g for 30 minutes with 4 cells each and employing unbuffered agar. A pair of films was prepared from the agar block of each cell, and 5 exposures were made with each film. The data of Table I show the individual variations encountered and indicate that values of considerable consistency can be obtained with the number of counts practical for routine studies.

Accuracy of the counts. The results obtained by sedimentation of the virus on agar have been compared with those seen with the spray procedure and on sedimentation of the virus on collodion-on-glass. The numbers of particles enumerated by the 3 methods are given in Table II.

A mixture was made containing 0.1 ml of the stock virus suspension, 0.45 ml H_2O, 0.75 ml of a 0.3% bovine serum albumin, and 1.2 ml of a water suspension of latex particles. This mixture resulted in a virus dilution of 1-25 and a salt content sufficiently low to yield clear micro-drop patterns when sprayed on collodion and subsequently shadowcast with chromium. Ten drops were photographed.

The number of latex particles per ml of stock suspension obtained by weighing was checked by sedimentation on collodion. Sedimentation gave a value in good agreement with that obtained by weighing (Table II). The value 6.2×10^{10}, derived by weighing, was used, together with the ratio of virus particles to polystyrene balls in the drop photographs, to calculate the virus content of the original stock from spray data with the mixture. It is seen that the results for virus count, obtained by both types of sedimentation technics, gave values in essential agreement with those yielded by the spray procedure. A somewhat higher, though still comparable, value resulted from calculations of particle number from the nitrogen content of the virus preparation.

Discussion. The data on the counting of influenza virus particles, such as those of Table II, show that essentially identical values were obtained with both the spray method and the procedures involving sedimentation. Only a limited number of factors would be expected to interfere with the accuracy of the spray technic, though absolute values are dependent on the accurate determination of the number of latex particles in the reference suspension and on thorough dispersion of both latex and virus particles in the test mixture.

A principal problem in counting with the sedimentation procedure is the control of the factors influencing the quantitative adherence of the particles to the receiving surface and transfer to the electron microscope. It is evident, however, as previously reported(2), and as found here, that conditions can be readily established for the counting of the influenza virus particles by sedimentation with the same results as those obtained by the spray method.

While the sedimentation technics are complex and tedious, the advantages which they offer, particularly through the use of the agar receiving surface, greatly broaden the field possible for investigation. The range of particle content optimum for accuracy with the sedimentation procedure is approximately 1,000-fold less than that needed for the spray technic. This far greater range brings within reach many tissue extracts and fluids and eliminates the need for either concentration or purification in those cases in which the virus can be readily recognized.

The special value of the agar surface resides in its applicability to preparations containing salt and, in common with the collodion surface, protein. Some viruses, for example those of Newcastle disease and erythromyeloblastic leucosis of fowls, are so distorted and unnatural when dried from preparations containing salt that recognition and counting of the particles are impossible. The advantages of the use of agar and of fixation with osmic acid in the electron micrography of these agents have been described(3). In that work it was observed that salt diffuses rapidly into the agar leaving no evidence of it detectable in the collodion film poured on the agar surface. In the present experiments there has been evidence that some protein may also diffuse into the agar block. Fixation of the virus with osmic acid while on the agar appeared to make the virus impervious to the effects of water during the immersion necessary for floating the membrane.

The applicability of sedimentation on agar to body fluids containing virus has been well illustrated in the case of the studies(3) on the erythromyeloblastic leucosis virus in the plasma from diseased fowls. More recently, preliminary investigations have indicated the feasibility of quantitative assay of influenza virus in chorioallantoic fluid from embryos diseased with this agent.

Summary. A method has been devised for counting virus particles in electron micrographs of the virus sedimented on an agar surface and removed quantitatively in a collodion pseudoreplica. With influenza virus A (PR8 strain) as test virus, the accuracy of the counts, under conditions practical for routine studies, was the same as that observed with the spray technic or by sedimentation of the agent on collodion. In common with the latter method, sedimentation on agar is applicable to materials of relatively low virus content and, since salt diffuses into the agar, to viruses harmed either by drying in the presence of salt or by salt-free water. The usefulness of this procedure is limited to the study of virus particles which can be recognized either because of characteristic morphology or of other properties that can be related quantitatively to virus behavior.

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A Technic for the Permanent Implantation of Electrodes on the Heart.[*]
(19783)

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Previous investigators(1-6) have studied the excitability cycle of the heart in acute open-chest experiments. After the sinus node was destroyed, a pair of electrodes was fixed for driving the heart, and another pair or the same pair was used to apply the testing shocks at different times in the cycle.

Since a more physiological preparation is desirable to obtain data on the excitability of the heart, a technic is presented which offers the following advantages: 1. Minimal tissue damage. 2. Fixed position of the electrodes in order to carry out repeated experiments in the same preparation under identical conditions. 3. Physiological condition of the preparation, *i.e.*, closed chest, spontaneous heart beat and respiration. 4. Current spread during stimulation is avoided by short distances (1 mm intervals) between the points of stimulation.

Technic. The technic for permanent implantation of multilead electrodes developed by one of us to study cerebral function has been used in the rat, cat, dog, and monkey (Delgado)(7). It has been modified to study the excitability of the heart.

Preparation of the electrodes. A piece of polyethylene tubing (No. PE 205, Clay Adams Co., Inc., New York) was opened and flattened to form a small plate 5 x 10 mm in size. A sharp needle lightly warmed was used to make 6 holes 1 mm apart in the midline of the plate. Six pieces of enameled stainless steel wire (Driver-Harris Company, Harrison, N. J.) approximately 6 inches in length and 0.12 mm in diameter were straightened by stretching. One end of each wire was melted to form a small ball (Riley)(8). The ends of the wire bearing the balls were threaded through the holes of the prepared plate. The other ends, coming to the opposite surface of the plate, were glued together by means of a thin solution of plexiglass. Thus only one surface of the plate presented the ball points. The wires were encased in polyethylene tubing 1.5 mm in diameter (No. PE 90).

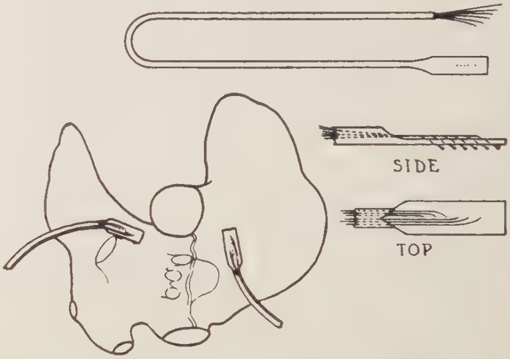

FIG. 1. Electrode is composed of 6 stainless steel wires isolated and protected by enamel, plexiglass and polyethylene. The plate is sutured to the epicardium.

The tubing near the plate and distal to the plate was fused by heat. In order to identify the points on the plate with the free ends of the wires, the latter were varied in length. The ball points on the plate were abraded with a fine emery wheel to make flat surfaces. The wires were checked for leakage with an ohmmeter. The electrodes made in this fashion (Fig. 1) were further prepared for implantation by making a small hole at each corner of the plate for subsequent suturing to the epicardium.

Surgical technic. Plate electrodes were sterilized in Zephiran (1:1000) over night. Dogs were anesthetized with nembutal, an endotracheal tube was inserted for positive pressure breathing. The chest was shaved and opened under aseptic conditions. Through an intercostal space (for auricular implantation the 3rd, and for ventricular the 5th, were most suitable) the pleura was opened and the ribs were retracted. The pericardium was slit over the site of the implantation and stay sutures were passed for retraction. With the aid of a 20 mm Carrel arterial needle, the sutures (000,000 silk) were inserted as follows: the needle was passed through one distal hole of the plate, then through the superficial epicardium, out the other distal hole, and tied over the plate. The proximal holes were then similarly treated. Thus the electrode was secured by only 2 sutures.

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The pericardium was closed and the free ends of the electrodes brought out through an interspace anterior or posterior to the incision. The ribs were approximated and a fine rubber tubing was temporarily inserted in the pleural space for subsequent evacuation of air. The chest wall was closed in layers, the intrapleural space evacuated, and the rubber tubing removed after which the skin was sutured.

The free end of the electrode outside the body was covered with a gauze square the edges of which were sealed to the skin by means of collodion, thus allowing easy access to the electrodes, and at the same time protecting them from breakage due to the animals' activities.

Comment. The results of studies using this technic have been reported(9). The presence of the electrodes does not interfere either with the movements nor with the health of the subject. Microscopic examination of the hearts of animals autopsied at varying intervals after implantation revealed no reaction except a slight thickening of the pericardium overlying the plate of the electrode.

The excitability determinations may be carried out by the methods described(10) without the use of any anesthetic agents provided the animals are trained to lie quietly. In addition to fulfilling the advantages stated above, this technic also makes it possible to study the conduction velocities of the different regions and the effects of pharmacological agents on both conduction velocities and excitability cycle determinations.

Summary. A technic has been described for the study of the excitability cycle of the heart employing permanent implantation of electrodes.

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Propagation of Coxsackie Virus on the Chorioallantoic Membrane of Embryonated Eggs.* (19784)

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In the course of efforts to determine convenient laboratory methods for differentiating

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between strains of herpes simplex virus and Coxsackie viruses isolated in suckling mice (1), the possibility was investigated that viruses of the Coxsackie group might propagate on the chorioallantoic membrane of embryonated hens' eggs. In previous attempts to grow Coxsackie or C viruses in eggs, positive results were obtained only with Type 2 strains and only when the yolk sac route of inoculation was employed(2,3). The capacity of herpes simplex virus to multiply on the chorioallantoic membrane has been well established. This agent resembles viruses of the Coxsackie group in that it causes a fatal disease in suckling mice and can be readily isolated in these animals(1,4-7). The purpose of this report is to present the results obtained with 17 strains of C virus, representing 16 different antigenic types, following inoculation on the chorioallantoic membrane. In this study, the technic employed with C viruses was similar to that commonly used for the propagation of herpes simplex virus.

Materials and methods. Viruses. The C viruses used were designated as follows: Types 1, 2, and 3(8); Texas-1, Texas-12, Texas-14, Texas-15, Ohio-1, Conn.-5, Israel-7, Easton-10, Easton-14 (strains L.O. and M.K.), Alaska-5, and Nancy(9); Belgium-1 and Belgium-2(10). The identity and specificity of each strain used were determined and confirmed by neutralization tests carried out in suckling mice with specific immune mouse or hamster sera. Two strains of the Easton-14 type were tested; otherwise, each of these agents was antigenically unrelated to the others. The Belgian strains were isolated in this labóratory from specimens collected during the summer of 1951 from patients in Brussels. Five of the other strains used were also recovered and identified in this laboratory.

Suckling mice. Suckling Swiss albino mice less than 48 hours of age were used for propagation of virus, for titrations of viral activity, for neutralization tests and in tests for the presence of virus in egg membranes. The number of baby mice in each litter was approximately eight.

Ten per cent suspensions consisting chiefly of muscle and bone were prepared in broth from infected suckling mice which had been killed with ether, decapitated, skinned and eviscerated. The suspensions were clarified by centrifugation first in an International Centrifuge at 2,500 RPM for 10 minutes and then in an angle rotor at 8,000 RPM for 10 minutes. The supernatant fluids prepared in this manner were treated with a mixture of penicillin and streptomycin to resist bacterial contamination and were used as a source of virus for the initial inoculation of egg membranes. Mice used for propagation of virus were injected with 0.02 ml of a suspension of infected baby mice by the intraperitoneal route and were sacrificed after signs of disease had appeared. Titrations of viral activity were carried out by injecting suckling mice by the intraperitoneal route with 0.02 ml of decimal dilutions of the various tissues to be tested. Tests for the presence of virus in egg membranes were performed by injecting suckling mice with 0.01 ml of a suspension of membranes by the intracerebral route. Following injection, the animals were observed daily during a period of 12 days for signs of disease or death. At the end of this period of observation the results were considered positive if all of the animals had developed fatal disease and negative if all of the animal remained healthy. Mice which died within 24 hours of the time of injection were discarded and not included in the test.

Embryonated eggs. Fertile hens' eggs which had been incubated at 36.6°C for 11 or 12 days were candled and those with active embryos were used in groups of 5 for each specimen or dilution tested. An inoculation of 0.1 ml was made on the chorioallantoic membrane of each egg. The eggs were then incubated again at 36.6°C for 72 hours. They were candled daily during this period and those in which the embryos had died were discarded.

The membranes of surviving embryos were harvested aseptically, washed in sterile saline, and ground in a mortar. They were then made into a 10% suspension in broth which was clarified by centrifugation for 10 minutes at 2,500 RPM. The suspensions were treated with a mixture of penicillin and streptomycin and stored in an electric refrigerator at —20°C. Cultures were made of all suspensions prior to inoculation of mice or eggs to

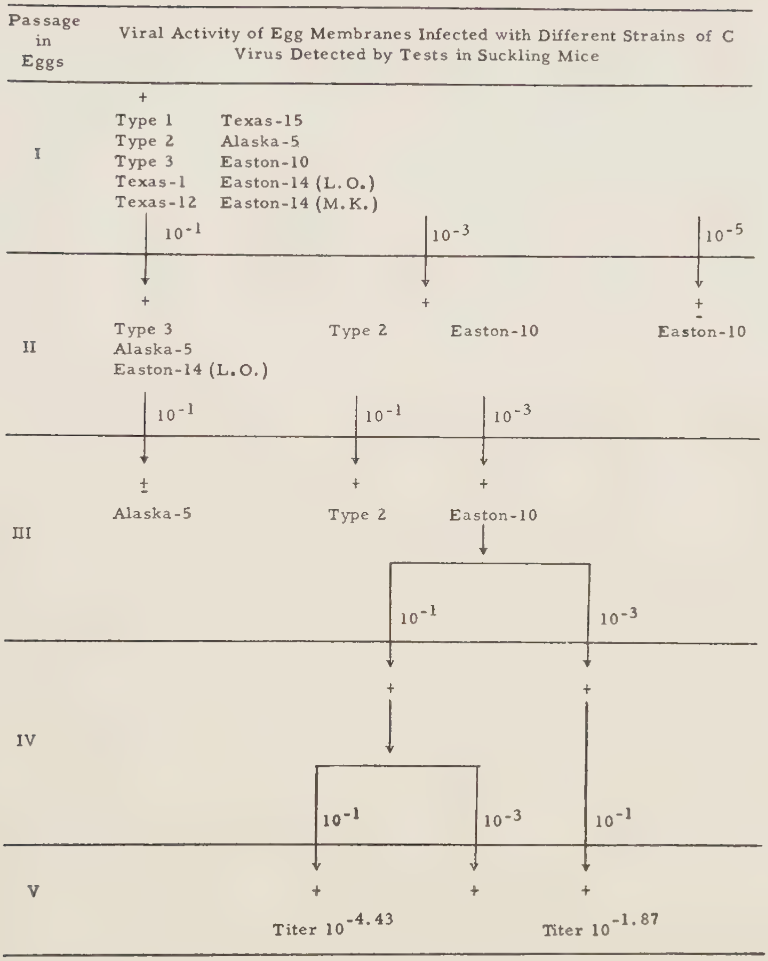

FIG. 1. Results obtained with 17 strains of 16 different antigenic types of C virus following inoculation on the chorioallantoic membrane of embryonated eggs.
Results: + = all of mice died; ± = approximately half of mice died.

assure absence of bacterial contamination.

An aliquot of each suspension of membrane was tested in suckling mice for viral activity. The occurrence of paralysis with fatal termination in these animals during the 12-day period of observation was taken to indicate the presence of virus. When a suspension of membranes was thus found to have viral activity, an additional serial passage was made in different groups of eggs using as inocula the 10% (10^{-1}) suspension and 2 additional dilutions (10^{-3} and 10^{-5}) calculated as dilutions of the egg membranes themselves. This was done in order to obtain as promptly as possible an indication of whether viral activity was attributable to a residue of virus from the original inoculum (passive transfer) or the result of actual multiplication of virus on the egg membrane. After the eggs used for this passage had been incubated for 72 hours, the membranes of each group were collected, made into a suspension and tested in suckling mice. Further passages in eggs were made in a similar manner with each of the membrane suspensions which was found to have viral activity. It should be emphasized that in this study all passages were made in eggs and that suckling mice were used merely as indicators in tests for the presence of virus.

Results. The results obtained with the 17 strains of C virus following inoculation on the chorioallantoic membranes of embryonated eggs are summarized in Fig. 1. The suspensions of infected baby mice which were used to inoculate first passage egg membranes had LD50 titers greater than 10^{-5} and less than 10^{-8} excepting suspensions of the Israel-7, Belgium-1 and Belgium-2 strains which had titers of 10^{-4} or less. In Fig. 1, only those strains which yielded positive results are indicated. Excepting the Easton-10 virus, each strain is recorded only once per passage in such a manner as to show the highest dilution of inoculum which transmitted viral activity. Higher dilutions and an additional passage of membranes which showed viral activity were tested but, except as indicated, the results were negative. Negative results were also obtained with suspensions prepared from uninoculated membranes and from membranes inoculated with sterile broth.

As can be seen in Fig. 1, after one egg passage, positive results were obtained in tests for viral activity with suspensions of membranes inoculated with each of 10 strains representing 9 different antigenic varieties of C virus. No evidence of viral activity was found in suspensions of membranes inoculated with the other 7 strains. In this and in subsequent passages most of the embryos remained alive during the 72 hours of incubation following inoculation. This was true both with eggs which showed viral activity and those which did not.

A second egg passage was made with each of the membrane suspensions which showed viral activity on the first passage. In tests for activity with membranes of the second passage, the results were positive with 5 strains and negative with 5 strains. Among the viruses which remained active on the second egg passage 3 were demonstrable only in membranes inoculated with a 10^{-1} suspension and not in those which received greater dilutions of the first passage material. The Type 2 and Easton-10 agents were present in membranes inoculated with a 10^{-1} and a 10^{-3} dilution, and some activity of the Easton-10 strain was also detected in membranes which had received a dilution of 10^{-5}.

In tests for the presence of virus in membranes of the third egg passage, the results were positive with the Type 2 and Easton-10 strains; some activity was also detected with the Alaska-5 virus. On the fourth egg passage only membranes inoculated with the Easton-10 strain were found to be active.

On the fifth consecutive egg passage of the Easton-10 strain, membranes inoculated with 3 different preparations of fourth passage material gave positive results in tests for the presence of virus. In order to confirm the identity of the viral agent in this passage one of the suspensions was tested with and found to be neutralized by specific immune mouse serum against the Easton-10 virus. Decimal dilutions of 2 of the fifth passage membrane suspensions were injected into suckling mice in order to estimate the amount of virus present in each of them. As indicated in Fig. 1, LD50 titers of $10^{-4.43}$ and $10^{-1.87}$ were obtained.

The suckling mouse material infected with the Easton-10 virus which was used to inoculate membranes of the first egg passage had an LD50 titer of $10^{-6.58}$. On the basis of dilutions made deliberately in the subsequent passages, quite apart from possible additional dilution within the eggs used, the inocula for the 3 groups of fifth passage membranes shown from left to right in Fig. 1 can be estimated to have contained as residue from the initial (first passage) inoculum not more than 0.01 LD50 for the first and 0.0001 LD50 for the second and third. The LD50 titers of $10^{-4.43}$ and $10^{-1.87}$ obtained with the first and third membrane suspensions of the fifth passage represent in both instances approximately one million times the calculated infective residue in the respective fifth passage inocula. These findings appear to indicate that in the serial egg passages, actual multiplication of the Easton-10 virus occurred.

Discussion. In this investigation it was found that the behavior of Coxsackie viruses following inoculation on the chorioallantoic membrane is not uniform and that several different antigenic varieties can remain active after more than one serial passage by this route. Variation in the quantity of virus contained in the suspensions used to inoculate membranes in the first egg passage and possibly also strain differences(3) may have affected the results which were obtained. In all but one instance, it seems possible to account for the presence of virus as residue from the initial inoculum. It is noteworthy, however, that the Type 2 virus, one of those which remained active through 3 consecutive egg passages is the only Coxsackie virus previously found to propagate in eggs(2,3).

Evidence was obtained that, in the case of the Easton-10 agent which remained active through 5 consecutive passages, actual multiplication of virus had occurred. The possibility that this agent might be a strain of herpes simplex virus and not a C virus seems unlikely in view of the procedures employed in its original isolation and identification(11) and would appear to be excluded by the results of other studies. These showed that, unlike herpes virus, the Easton-10 agent was resistant to treatment with ether and failed to cause apparent disease in adult mice inoculated by the intracerebral route or lesions following inoculation on the scarified cornea of the rabbit's eye(1).

It should be pointed out that in the present study possible variations in the procedure used were not explored. Only embryos of 11 or 12 days of age, only one route of inoculation, and only one interval of incubation following inoculation were employed. The chorioallantoic membrane was the only component of the embryonated egg which was tested for the presence of virus. Optimal conditions for the propagation of Coxsackie viruses in eggs were not investigated. It is quite possible that for this latter purpose better results could be obtained by varying the procedures which were used.

In view of the dissimilar results obtained with different Coxsackie viruses, the technic of inoculation on the chorioallantoic membrane appears to be of limited value for the purpose of differentiating between these agents and herpes simplex virus.

Summary. Seventeen strains of C virus representing 16 different antigenic varieties were tested for their capacity to remain active or multiply following consecutive passages in embryonated eggs inoculated on the chorioallantoic membrane. Viral activity was demonstrated in suckling mice with membranes infected by each of 10 strains after one egg passage, by 5 strains after an additional passage, by 3 strains after 3 passages and by one strain (Easton-10) after 5 passages. The findings indicated that multiplication of the Easton-10 virus had occurred.

The assistance of Miss Eru Tanabe is gratefully acknowledged. Dr. J. L. Melnick kindly provided the Easton-10 strain of virus and homologous antiserum as well as some of the other strains and immune sera used in this study.

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Experimental Lathyrism. (19785)

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Previous reports from this laboratory have been concerned with experimental lathyrism in rats and mice(1,2). We now report additional investigations with 3 of the species of *Lathyrus* previously studied (*L. odoratus, L. sylvestris Wagneri,* and *L. latifolius*), and with 2 new species,[†] *L. splendens* (the campo pea) and *L. strictus*. Preliminary studies of the concentration of the noxious principle of *L. sylvestris Wagneri* are reported. This species in our experience has shown itself to be the most toxic of all the species of *Lathyrus* studied.

The diets and general plan of the experiments were the same as those of earlier studies (1,2). The ground peas comprised 50% of the diet with 10% of casein included to provide an adequate amount of an animal protein of good quality.

The seed of *L. splendens* was found to be of about the same toxicity for white rats as *L. latifolius*(2). Four rats with an average initial weight of 92 g died in 5 to 11 days after consuming an average of 15 g of the legume. The symptoms produced, however, were quite different in that no hyperirritability or convulsive seizures so characteristic of rats fed *L. sylvestris Wagneri* or *latifolius* were noted.

TABLE I. Relative Toxicity of *Lathyrus* Legumes for White Rat and White Mouse.

Lathyrus species	Fatal dosage*	Relative toxicity†
Rat		
sylvestris Wagneri	5.8	100
splendens	20.7	28
latifolius	22.3	26
odoratus	289	2
Mouse		
sylvestris Wagneri	25.4	100
latifolius	32.8	78

* Amount of pea per 100 g body wt which produced death.

† The toxicity of *L. sylvestris Wagneri* is arbitrarily designated as 100.

TABLE II. Toxicity of *L. sylvestris Wagneri* and *L. latifolius* to Different Species.

	No. of animals	Fatal dosage*	Ratio with the rat as unity
Lathyrus sylvestris Wagneri			
Guinea pig	3	3.3	.57
Rat	14	5.8	1
Rabbit	3	6.7	1.15
Mouse	3	25.4	4.37
Lathyrus latifolius			
Rat	4	22.3	1
Mouse	3	32.8	1.47

* Amount of pea per 100 g body wt necessary to produce death.

The experiments with *L. strictus*[‡] were limited by the small amount of the seed of this species available. Three rats of approximately 120 g initial weight consumed 112, 104 and 115 g of the diet respectively over a period of 14 days (equivalent to approximately 55 g of the legume). During this period the changes in weight were –3.5, –13.9, and +2.3 g as compared with +11.7, +2.5 and +19.7 g in pair-fed litter mate controls which received the edible white split pea of commerce. No evidence of toxicity was observed, apart from the failure to gain as did the control rats. It is evident that any toxicity of *L. strictus* was definitely less than that of *sylvestris Wagneri, splendens,* or *latifolius*. However, it was more toxic (as shown by losses in weight) than *odoratus, sativus,* or *hirsutus*(1).

A comparison of the toxicities of the 4 chief species of *Lathyrus* studied on the basis of the amount of the legume eaten per 100 g of body weight is given in Table I. It is seen that *L. sylvestris Wagneri* is 3 to 4 times more toxic than *splendens* or *latifolius* and about 50 times more toxic than *odoratus* for the rat.

Intoxication due to *L. sylvestris Wagneri* was also studied in other species of animals

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[*] These experiments were supported in part by a grant from the Rackham Research Funds of the Horace H. Rackham Graduate School of the University of Michigan.

[†] Seed of *Lathyrus strictus* was obtained from Mrs. E. W. Cisler of San Diego, Calif., and of *L. splendens* from Theodore Payne of Los Angeles with the cooperation of Mrs. Cisler.

[‡] These experiments were conducted by Mr. T. R. Sawyer as a research assistant.

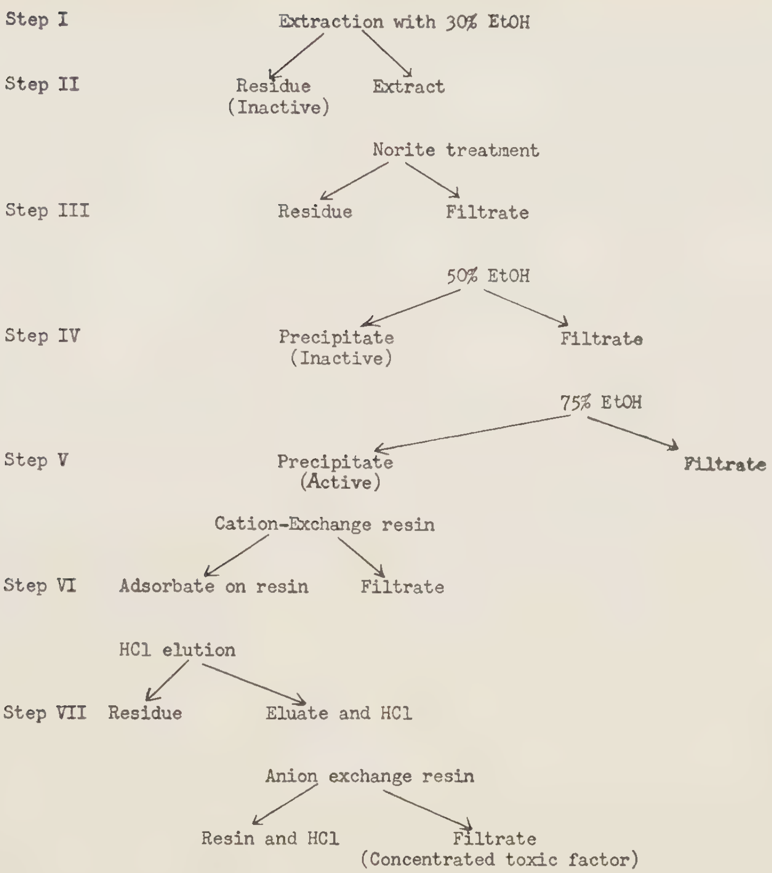

FIG. 1. Scheme of fractionation.

(Table II). It is known that there is a great species variation of toxicity in animals receiving *Lathyrus,* notably among domestic animals (3). Essentially the same amount of legume per 100 g of body weight was required as the fatal dose in the rabbit as in the rat. Death occurred when the guinea pig had consumed only 60% of this amount, while 4 times this amount of *sylvestris Wagneri* was required to produce fatal toxicity in the mouse. The mouse also proved more resistant to intoxication by *latifolius* than the rat (Table II).

A procedure, devised for the concentration of the toxic factor in *L. sylvestris Wagneri,* is detailed to Fig. 1. Step I is the extraction of the toxic principle from the finely ground legume with 30% ethanol, as previously reported(1), an extraction which removed approximately 20% of the solids. This extract is dried *in vacuo* at 50 to 60°, incorporated into a 10% aqueous solution, mixed with Norite (10 g per 1 of solution), shaken for one hr, and the solution is filtered. About 35% of the solids are removed with the Norite; the toxic principle remains in the filtrate. In step III, an equal volume of ethanol is added to this filtrate, which results in the precipitation of a small amount on an inactive fraction (less than 1% of the total solids). When the concentration of ethanol is increased to 75%, approximately half of the total solids is precipitated; this fraction contains the greatest part of the toxic principle. After filtration, the precipitate is dissolved to give a 2% aqueous solution and passed through a cation-exchange column.[§] The resin

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[§] The Rohm and Haas cation-exchange resin IR-100-H AG was employed. The recommendations contained in their laboratory manual were followed. Columns of 22 mm internal diameter and 120 cm long filled to a height of approximately 70 cm with 150 g of resin were used.

TABLE III. Concentration of Toxic Factor of *Lathyrus sylvestris Wagneri.*

No. of rats	Avg wt, g	Treatment	Fatal dosage/100 g rat, g	Concentration	
				In single step	Over all
14	58.7	None	5.8	—	—
4	61.4	Extn. with 30% EtOH	1.8	3.22	3.22
3	76.2	Norite treatment	.85	2.12	6.82
6	57.8	EtOH pptn. Column absorption and elution	.49	1.73	11.84
4	51.4	2% acid elute	.35	1.40	16.6
4	53.1	4% " "	.145	3.38	40

adsorbs about 60% of the solids from the solution; the toxic principle is contained in the adsorbed fraction. Step VI involves the elution of the adsorbed material by hydrochloric acid. The separation and concentration is completed by the removal of the acid by ion exchange resin IR-4B.

The acid eluates from Step VI were found to vary in toxicity, depending upon the amount and strength of the acid used. Weak acid (1% or less) elutes material of low toxicity, whereas subsequent elution with stronger acid (2-4%) gives highly toxic fractions. For example, in one run, 15 g of material was adsorbed onto the resin column (Step V) and serially eluted with approximately 1 l each of 0.5, 1, 2, and 4% hydrochloric acid. The weakest acid (0.5%) eluted 8 g from the column, while the 3 higher concentrations eluted about 2.3 g each. The amounts of each fraction which effected the death of young white rats (approximately 60 g in weight) were 0.68, 0.32, 0.19 and 0.08 g respectively.

Toxicity of each fraction was assayed by feeding to the rat with the basal white pea diet and determining the fatal dosage. The concentration of the toxic factor which has been effected at each step in a typical experiment is shown in Table III. In these data the fatal dose is calculated in terms of 100 g body weight.

A total of 10 species of *Lathyrus* has now been studied in our laboratory in relation to the nutritive value and toxicity of these leguminous seeds for the young white rat. Of these, *Lathyrus sativus* and *cicera* (reported to be of clinical importance in the lathyrism of man) proved to be relatively non-toxic to the young white rat and supported moderately good growth over considerable periods of time, growth comparable with that observed with *Pisum sativum,* the edible dried pea of commerce. On the other hand, the toxicity of *sylvestris Wagneri* was greatest of all the species of *Lathyrus* examined; this was true for all the species of animals to which it was fed (white rat, white mouse, rabbit, guinea pig). Of the other species, *latifolius* and *splendens* were extremely toxic, as was also *tingitanus* to a slightly lesser degree. Experimental lathyrism with marked changes in bones(1) and related structures was readily produced in 6-12 weeks with *odoratus* and in approximately 16 weeks with *hirsutus.* It would seem highly improbable that the marked skeletal changes observed with these 2 species would be produced by species which were so toxic as to induce death within a few days (*sylvestris Wagneri, latifolius, splendens*). The toxicity of the first 2 of these was associated with characteristic nervous symptoms (marked irritability and convulsion) which became progressively more acute until death ensued. There were slight variations in toxicity in relation to the species of animal, but with one exception the differences were not marked. However it was not possible to observe the toxicity and skeletal changes in mice fed *odoratus* such as were seen in rats.

The nature of the toxic principle of the various species of *Lathyrus* is not known. In all the species which we have studied, extraction with cold water left a relatively non-toxic residue; ether failed to remove the toxic agent. For practical purposes, we have found that 30% alcohol is the most effective extractant. From such an alcoholic extract of *Lathyrus sylvestris Wagneri,* we have been able to effect an approximately 40-fold concentration of the toxic material by absorption on a cation-exchange resin, from which the toxicant could be eluted by 2-4% hydrochloric acid. In view of the interest in lathyrism, experimental and clinical, we are reporting these observations, since there is no prospect that we can extend our investigations of this subject in the immediate future.

Summary. A comparison of the toxicity of the seed of 5 species of *Lathyrus* for the rat has been made. Intoxication with *L. sylvestris Wagneri* has also been studied in the white mouse, rabbit, and guinea pig. A partial purification of the toxic principle of *L. sylvestris Wagneri* which has concentrated the factor about 40 times is described.

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Renal Transport of Para-Aminohippurate in the Hypophysectomized Rat.* (19786)

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A number of investigators have shown that the clearance of inulin, diodrast and para-aminohippurate as well as the tubular transport maximum (Tm) of para-aminohippurate and glucose are reduced in hypophysectomized animals(1-5). The reduction in Tm as determined with para-aminohippurate (PAH Tm) could be due to a reduction in the renal blood flow or due to a defect in the tubular transport of para-aminohippurate. The latter point can be readily studied by the para-aminohippurate concentration technic of Cross and Taggart(6) in kidney slices.

Methods. Male, Sprague Dawley rats, 30-36 days following hypophysectomy† were used in this study; litter mates were used as controls. The hypophysectomized rats weighed 89 to 97 g while the control litter mates weighed 110 to 230 g. A series of hypophysectomized rats were injected with the equivalent of 1 mg of growth hormone‡ (Armour standard) per 100 g of body weight twice daily for 5 days. Two growth hormone preparations (Armour & Co.)‡ prepared by the method of Wilhelmi, *et al.*(7) were administered to each of 2 groups of hypophysectomized rats. Kidney slices were prepared by conventional methods and incubated in a buffer similar to that of Cross and Taggart(6). The PAH concentration used was 0.0013 molar. The weight of the incubated slices varied between 55 to 80 mg. One set of vessels contained no substrate while the other set contained 0.01 molar sodium acetate. The vessels were incubated for 2 hours at 25°C in an oxygen atmosphere in a Dubnoff metabolic shaking incubator. PAH determinations in the slice and medium were determined by the same methods as those used by Cross and Taggart(6).

Results. Cross and Taggart have expressed the PAH concentrating ability of kidney slices as the slice medium PAH concentration ratio (SM ratio). In the present series of experiments, the SM ratio will be used as an index of activity of the renal slices.

Table I summarizes our results obtained so far and it is clear that the kidney slices from hypophysectomized rats have a much lower SM ratio than slices from control rats. The administration of growth hormone for 5 days increased the body weight of the rats by about 24 grams and the para-aminohippurate SM

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† Hypophysectomized rats were supplied by Hormone Assay Laboratory, Chicago, Ill.

‡ Kindly supplied by Mr. Irby Bunding, Armour and Co., Chicago, Ill.

TABLE I. Effect of Hypophysectomy and Growth Hormone Treatment on Para-aminohippurate Slice Medium Ratio of Rat Kidney Slices. Values are slice medium ratio of para-aminohippurate, and standard deviation. Substrate when used was .01 molar sodium acetate.

Rats	No. of rats	Wt of rats, g	S/M ratio for PAH	
			No substrate	With substrate
Normal control	6	218–230	9.71 ± 1.00	14.48 ± 1.36
" "	6	110–120	9.16 ± 1.58	12.45 ± 1.36
Hypophysectomized	6	90–97	5.36 ± 1.17	6.38 ± 2.26
Hypophysectomized, treated with growth hormone*	6	92–96	10.68 ± 1.69	14.07 ± 1.33
Hypophysectomized, treated with growth hormone†	9	89–96	9.98 ± 2.11	15.76 ± 3.62
Normal, treated with growth hormone†	6	215–239	9.50 ± 1.98	13.54 ± 3.47

* Growth hormone, Armour & Co., Lot No. K40805R.
† R377237.

ratio of the kidney slices to normal levels. Furthermore, it can be seen that with normal kidney slices the addition of acetate increased the SM ratio by about 40-50%; with kidney slices from hypophysectomized animals the stimulating effect of acetate is very small. Growth hormone administration increased the SM ratio in the absence of substrate and also restored the ability of the kidney slices to respond to acetate. The administration of growth hormone to normal rats weighing 200 to 250 g in the same dosage as that used in the hypophysectomized rats did not have any significant effects on the SM ratio of the kidney slices.

Discussion and conclusion. The reduction in the SM ratio of kidney slices from hypophysectomized animals may be due to the reduction of some rate limiting factor in the kidney. Cross and Taggart(6) and Mudge and Taggart(8) have postulated that acetate or the hypothetical two carbon fragment may be a rate limiting factor. However, our results clearly indicate that in the present instance this is not the case since addition of acetate did not restore the ability of kidney slices from hypophysectomized rats to concentrate para-aminohippurate. In hypophysectomized rats, Qo_2, as well as alkaline phosphatase activity of the kidney are less than normal(9). It is possible that this enzyme may be important for kidney function and the probable defect may be a reduced concentration of this enzyme or some other rate limiting enzyme system essential for PAH transport. Growth hormone which produces nitrogen retention may well stimulate the production of the enzyme or enzyme systems necessary for PAH transport. The above findings may in part explain the restorative effects of growth hormone on the PAH Tm of the hypophysectomized animals. Although the restoration of the SM ratio was produced by growth hormone, the possibility remains that this restorative action may have been produced by some contaminant of the hormone preparations used.

Summary. The uptake of para-aminohippurate of rat kidney slices was determined. Kidney slices from hypophysectomized rats show a lower ability to concentrate para-aminohippurate than normal rat kidney slices. This defect is corrected by the prior administration of growth hormone to the hypophysectomized rats.

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Cultivation of *Coccidioides immitis* in the Developing Hen's Egg. (19787)

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The tissue, or sporangial, phase of *Coccidioides immitis* has been reported capable of forming in developing hen's eggs. The first report described the appearance of the tissue phase after inoculation of the mycelial phase onto the chorioallantoic membrane(4). The formation of the sporangia in embryo, albumen, yolk and embryonic fluids was also reported(3). Brueck and Buddingh(2) were unable to repeat the above work, but noted the ready formation of the tissue phase in the yolk of developing chick embryos.

In view of these contradictory results, investigations were initiated to determine under what conditions maximum numbers of sporangia developed in the fertile hen's egg. In this paper, experiments are described relating to the optimum age for inoculation of the fertile hen's eggs, the death rate of the inoculated eggs, and the formation of the tissue phase.

Methods. Three- to 12-day-old embryos were chosen and inoculated via either the yolk sac, allantoic sac, or the chorioallantoic membrane routes. The methods employed were essentially those described by Beveridge and Burnet(1). The yolk sac inoculations were made through a small hole punched in the center of the broad end of the egg. Through this a 1¼-inch, 22 gauge, hypodermic needle was thrust past the air sac into the yolk sac. The allantoic sac inoculations were made with a similar needle and through a similar hole punched in the side of the broad end of the egg, along the margin of the air sac. The needle was inserted only a short distance as compared with the full length insertion for the yolk sac inoculation. The inoculum for both routes of inoculation consisted of a 0.1 ml suspension of *C. immitis* spores and hyphal elements in physiologic saline. The chorion side of the chorioallantois was inoculated, using the dropped membrane technic and the same inoculum (0.1 ml) as above. The openings were sealed with a collodion-iodine mixture and the eggs incubated at 96° or 98°F. The eggs were candled twice daily. Physiologic saline suspensions of mature spore-forming cultures of *C. immitis,* strain No. 2150, received from Dr. Norman F. Conant, were used throughout the project.

Results. Effect of embryo age on mortality after infection. The mortality of the inoculated eggs depends more upon the age of the embryo than the route of inoculation (Table I). In developing eggs inoculated via the yolk sac, allantoic sac, or chorioallantoic membrane, 3-, 5-, 7-, and 8-day-old eggs showed an especially high mortality. By the end of the first 5 days of incubation all the embryos in the 3-day series were dead. Dead, also, were 91% of the 5-day eggs, 80% of the 7-day eggs, and 73% of the 8-day eggs. Since smears and cultures were made of eggs from all series, it was possible to rule out secondary bacterial infections as being of importance in these mortality figures. In contrast to 3- to 8-day eggs, the 9- to 12-day eggs showed a low mortality. Of the 9- to 12-day eggs examined during the same 5-day period after inoculation, only 24, 19, 15, and 7%, respectively, died (Table I). Throughout this 5-day period several live eggs were removed for examination. It should be realized then, that these percentages are approximate, and possibly would have been different had these living eggs not been selected for study.

In yolk sac inoculations, the number of deaths for eggs of all ages was low on the first day after infection, ranging from 0 to 12%. This may represent partly nonspecific deaths. Thereafter, the number of deaths increased markedly, reaching a peak on the third day in the 3-, 5-, 7-, and 8-day-old eggs. A much lower peak was noted on the second day, maintained through the third day in 9-day-old eggs.

In the series of eggs inoculated via the

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TABLE I. Mortality of 3- to 12-Day-Old Developing Hens' Eggs after Inoculation with Standard Spore Suspension of *C. immitis* via One of Several Routes.

Age of eggs, days	No. inoculated and route	No. eggs dying 1st-5th day					Total No. dead	% dead end of 5 days' incubation
		1st	2nd	3rd	4th	5th		
3	22 YS*	0	8	13	1		22/22†	100
5	45 "	1	6	33	1		41/45	91
7	56 "	2	7	27	7	2	45/56	80
	25 AS	0	2	7	10	1	20/25	80
8	275 YS	27	44	85	41	4	201/275	73
	60 C-A m	11	8	10	13	2	44/60	73
9	49 YS	2	4	4	2		12/49	24
10	26 "	3	1	0	1		5/26	19
11	44 "	3	1	2	1		7/44	15
12	28 "	1	0	0	1		2/28	7
Total	630							

* YS = yolk sac. AS = allantoic sac. m = membrane.

† Numerator indicates total number of eggs dead; denominator, total number of eggs inoculated.

allantoic cavity no deaths were evident on the first day after infection; whereas, in the series inoculated onto the chorioallantoic membrane 18% deaths were noted. There is some evidence in these 2 series of eggs that the greatest number of deaths occurred on the fourth day, in contrast to the third day in yolk sac inoculations. However, the overall mortality in the 5-day incubation period did not differ appreciably from that of the yolk sac group.

From the data of Table I, it seems that 3- to 8-day old embryos were more susceptible to infection by *C. immitis* than 9- to 12-day-old embryos, in that a higher mortality occurred in shorter periods of time. For studies of the formation of the tissue phase, however, it may be disadvantageous to have a host with a short survival time after inoculation, especially if more than 5 days are required for ready observations of the sporangia. However, it was increasingly difficult to observe the fungus in the older (9- to 12-day) eggs. Hence, studies of the tissue phase were largely but not entirely limited to 8-day-old eggs.‡

Distribution of C. immitis in eggs. The yolk sac, allantoic sac, and chorioallantoic membrane were inoculated with suspensions of spores and short hyphal elements in physiologic saline. The resulting infection was followed by examining fresh smears of the yolk, allantoic fluid, amniotic fluid, and when indicated, albumen. Both living and dead eggs of all available eggs were studied daily.

As studied in smears, in the yolk sac inoculations the mycelial phase increased during the first 3-4 days after infection. On the third day, 30% of the living eggs, and an even slightly larger number of the dead eggs, showed mycelium in the yolk fluid. This percentage decreased after the fourth day, and it was often difficult to demonstrate *C. immitis* mycelium on the fifth, sixth, and seventh days after infection. Some smears of the fourth and later days showed mycelium which appeared to be degenerated.

In the 2 series of eggs inoculated in the allantois and onto the chorioallantois, it was unusual to find mycelium at the site of inoculation after the second day of incubation. However, if the air sac became infected mycelium was more likely to be found in the allantoic fluid. One striking feature of the allantoic and chorioallantoic inoculations was that mycelium was often found in the yolk by the third day, a phenomenon which indicated that the fungus could penetrate the extra-embryonic membranes and yolk sac, and also that the yolk fluid was probably a preferable medium in the egg.

Regardless which of the 3 routes of inoculation was used, mycelium often could be found growing on the air sac membrane, and was usually present on both surfaces. Such colonies might have arisen as the result of contamination in the case of yolk sac inoculations and from extension in the case of allantois and chorioallantoic-membrane inoculations.

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‡ In a recent paper, published since the completion of this study, R. A. Vogel and N. F. Conant have shown that sporangia could be obtained in abundance by serial inoculation of the yolk sac of 10-day-old embryos. PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 544.

Formation of sporangia. Sporangia, or sporangia-like, bodies have been found to be produced by some *C. immitis* mycelium during the third, fourth, and fifth days of incubation. Whether these bodies were formed at a later period is not known, for few infected older eggs were available for study. The sporangia-like bodies occurred in yolk fluid both of living and of dead eggs. In a few instances, slides which were prepared of positive (mycelium) yolk were found to contain some sporangial initials after being sealed and placed in a slide box for 2-7 days. These sporangial initials were formed after the slide mount was prepared. No sporangia were observed in positive yolk fluid transferred to test tubes, layered with mineral oil and incubated at 37°C and room temperature.

Most of the sporangia-like bodies observed were formed by the mycelium. A few, unattached to the mycelium, may have arisen from spores or short hyphal filaments. It was often difficult to be certain of the identity of these free cells, since some of the normal components of yolk fluid resemble them so closely. The sporangial initials were formed intercalarily, laterally, and terminally. They began as swellings, often uneven in appearance, which contained a thin granular cytoplasm. Vacuoles were formed in this cytoplasm early in the development of the cell and could be seen to move about, increase and diminish in size. The cell wall appeared to remain plastic for a considerable period of time, and was thin-walled compared with that of the sporangia formed in animal tissue. Eventually, cross walls were developed to demarcate the sporangial initial from the mycelium. What has been interpreted as cleavage was a rapid process requiring but a few minutes. The cleaved cytoplasm rounded into endospore-like bodies which usually were of similar size; these increased somewhat in diameter. Mature organisms varied greatly in size, but most averaged about 10-15 μ in diameter.

Discussion. It seems that in developing hen's eggs, yolk is a more satisfactory medium for the formation of the tissue phase than allantoic fluid, amniotic fluid or albumen. As demonstrated by smears, sporangia are formed in some eggs by the end of the fifth day of incubation; however, these sporangia are often few in number and sometimes difficult to differentiate from the normal components of the yolk.

It appears that the mortality of the inoculated eggs is more dependent upon the age of the embryo than on the route of inoculation. This is well brought out by the figures in Table I, which indicates a striking decrease in mortality with corresponding increase in age of inoculated embryos. The percentage deaths of 7- and 8-day-old embryos in eggs inoculated via the allantoic and chorioallantoic membrane was almost identical with the percentage of deaths of the same age groups inoculated via the yolk sac.

The basis for the marked decrease in mortality and apparent susceptibility to infection of eggs 9 days of age and older has not been investigated. The 70%, or greater, mortality of 3- to 8-day eggs in a 5-day period is decidedly higher than the mortality for 9- to 12-day-old eggs, which is less than 25%. The high mortality rate of the 3- to 8-day eggs may make this age group most suitable for testing in chemotherapeutic studies of coccidioidomycosis.

Summary. 1. Six hundred and thirty 3- to 12-day chick embryos were inoculated with a suspension of the mycelial phase of *C. immitis* in physiologic saline, via the yolk sac, allantoic sac, or chorioallantoic membrane routes. 2. The mortality of the inoculated eggs depended more upon the age of the embryo than the route of inoculation: 3- to 8-day embryos showed a mortality of 100-73% in the 5-day period of incubation; whereas, 9- to 12-day embryos showed a mortality of only 24-7%. 3. The distribution of *C. immitis* in inoculated eggs was studied and the formation of sporangia, or sporangia-like, bodies noted. These

bodies are few in number and difficult to interpret.

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Antigenic Similarity Between the First and Later Egg Passage Strains of Freshly Recovered Influenza A Viruses. (19788)

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Analysis of the significance of antigenic variation of influenza viruses in man depends in part on the validity of the assumption that the antigenic properties of strains recovered in the laboratory in unnatural hosts correspond to those of the agents infecting human beings. From this point of view, the use of mice for the recovery or serial passage of influenza viruses appears to be highly undesirable for it is known(1,2) that these agents may show unpredictable changes in antigenic composition when maintained in this host. On the other hand, there is evidence(3) that recently recovered influenza virus strains may be maintained for 6-12 serial passages in the chick embryo without undergoing antigenic variation. The possibility that a change in antigenic composition may occur immediately after recovery during the first few passages in the chick embryo has not been investigated previously.

The present study was undertaken in order to determine whether, after serial embryo passage, influenza virus strains are antigenically similar to or different from the same strains in the 1st egg passage on recovery from throat washings.

Materials and methods. Recovery and serial passage of viruses. During the 1951 epidemic in New York, a number of influenza A virus strains were recovered by inoculation of chick embryos in this laboratory. On the basis of the extent of multiplication obtained during the 1st embryo passage, throat washings from 2 patients (NA and RA) with influenza A were chosen for further study. On 4 different attempts with each throat washing, infection was induced in a considerable proportion of embryos inoculated. The strains of each virus used in the experiments described below were obtained as follows:

NA. 10-day-old chick embryos were inoculated amniotically with 0.2 ml of throat washings from patient NA which contained penicillin, 250 μ, and streptomycin, 2500 μg/ml. After incubation at 35°C for 70 hours, followed by chilling at 4°C overnight, allantoic and amniotic fluids were harvested separately. Agglutination tests with 1% guinea pig RBC (0.5% final) were carried out with fluids from individual eggs employing a final dilution of 1:4. Of 57 amniotic fluids, 12 were positive. All allantoic fluids from these eggs were negative. The hemagglutination titer of the pool of positive amniotic fluids at 24.5°C was 1:64 with guinea pig RBC and 1:8 with chicken RBC. For the preparation of antisera, the pool of positive amniotic fluids was diluted with 5 volumes of amniotic fluids from the same 1st passage eggs which had given negative hemagglutination reactions. 7 serial allantoic passages were carried out in 10- to 11-day chick embryos, starting with a 10^{-1} dilution of the 1st passage amniotic fluid pool and continuing with a 10^{-3} dilution of allantoic fluid at each subsequent passage. Eggs were incubated at 35°C for 48 hours, chilled at 4°C overnight and the allantoic fluids were harvested, pooled, and stored at –65°C.

RA. Primary recovery of this strain from the throat washing of patient RA was carried out in the allantoic sac. The 1st passage strain was prepared in 2 batches which then were pooled. The 1st batch was obtained by inoculating 28 chick embryos intra-allantoically with 0.2 ml of throat washing containing penicillin and streptomycin as above. After incubation at 35°C for 96 hours, allantoic fluids from 8 embryos showed a positive hemagglutination reaction at a final dilution of 1:4 with guinea pig RBC. With the 2nd batch, allantoic fluids from 12 of 72 embryos handled in an identical manner were similarly positive. Only the positive allantoic fluids from the 2 batches were pooled. Hemagglutination titrations carried out at 24.5°C with the positive fluids from each batch showed titers of 1:64 with guinea pig RBC and 1:16 with chicken RBC. 4 serial allantoic passages then were made in the manner described above.

Virus titrations. Hemagglutination and infectivity titrations *in ovo* were carried out by technics identical with those described previously(4,5).

Immune sera. Roosters and rabbits were injected intravenously with 10 ml of infected amniotic or allantoic fluid pools. Two animals were used for each virus preparation. Roosters were bled 26 days later, and a 2nd intravenous injection of virus was then given. The final bleeding was obtained 46 days after the 1st injection. Rabbits were bled 21 days after the 1st injection.

Antibody titrations. Hemagglutination-inhibition and *in ovo* neutralization titrations were carried out with 2-fold dilutions of inactivated serum by the technics described previously(5). Approximately 1,000 EI_{50} of virus was employed in each of the neutralization tests. Serum antibody titer ratios for heterologous sera were computed as described in a prior paper(3). In computing such ratios, the reciprocals of the titers were used.

Experimental. Identification of NA and RA strains. Hemagglutination-inhibition titrations with the 2 strains were performed with rabbit antisera against various influenza viruses. Guinea pig RBC were employed. The sera were heated at 56°C for 30 minutes, absorbed with 10% guinea pig RBC and treated with PR8 or Lee virus at 42°C for 3 hours as described previously(6) to remove nonspecific inhibitor. The treated sera were then heated at 65°C for 30 minutes to inactivate the added virus. This procedure yielded sera which caused neither agglutination of guinea pig RBC nor nonspecific inhibition of influenza virus hemagglutination.

TABLE I. Identification of NA and RA Strains by the Hemagglutination-Inhibition Procedure.

Rabbit serum vs.	Hemagglutination-inhibition titer* Virus strain NA†	RA‡
Normal	<16	<16
PR8	<16	<16
FM1	512	512
MA§	256	256
Lee	<16	<16
MB‖		
1233		

* Titer expressed as reciprocal of dilution at end point.

† Infected allantoic fluid, 2nd passage from throat washing.

‡ Infected allantoic fluid, 1st passage from throat washing.

§ 1950 influenza A virus strain.

‖ " " B " " (6).

The results of hemagglutination-inhibition titrations with the 2 strains at the 1st or 2nd embryo passage are given in Table I. As can be seen, both the NA and RA strains were inhibited by rabbit antisera against FM1 (1947) and MA (1950) influenza A virus strains, but not by antiserum against the PR8 strain. Antisera against influenza B and C (strain 1233) viruses showed no inhibitory effect.

Hemagglutination - inhibiting antibodies against the homologous and a number of heterologous influenza virus strains were measured in the acute and convalescent sera of the patients from whom the NA and RA strains were recovered. Guinea pig RBC were used. The sera were diluted with equal volumes of buffered saline and heated at 65°C for 30 minutes. Table II summarizes the results of these antibody titrations. Both patients showed a rise in antibodies against their own strain as well as against FM1 and PR8, but not against Lee or 1233.

Changes in titer of NA and RA strains during early passages. The titers of the NA and RA strains during early passages in the chick embryo were followed in terms of infectivity *in ovo* and hemagglutinating capacity with

TABLE II. Hemagglutination-Inhibiting Antibody Rise in Patients from Whom NA and RA Strains Were Recovered.

		Hemagglutination-inhibition titer						
		Virus strain						
Patient's serum		NA	RA	MA	FM1	PR8	Lee	1233
NA	Acute	<16	<16	<16	32	64	32	256
	Convalescent	32	<16	<16	256	128	32	256
RA	Acute	<16	<16	<16	16	32	32	256
	Convalescent	64	64	64	256	128	32	256

Guinea pig RBC were used except with 1233 in which case chicken RBC were used. 8 units final of each virus was employed.

TABLE III. Increase in Infectivity Titers *In Ovo* of NA and RA Strains During Early Passage.

	Embryo passage No.		
Virus strain	Amniotic sac	Allantoic sac	Infectivity titer* EI_{50}, log
NA	1		–5.3
	1	1	–8
	1	7	–7.7
RA		1	–5.5
		2	–6.3
		5	–7.7

* Geometric mean of 2 titrations.

both guinea pig and chicken RBC. Table III summarizes the results of infectivity titrations carried out in the allantoic sac of embryonated eggs. The NA strain reached maximal infectivity for the chicken embryo after 1 amniotic and 1 allantoic passage whereas the RA strain, which was recovered directly in the allantoic sac, did not show a similar high titer at the 2nd allantoic passage but did so at the 5th passage. Table IV gives the results of hemagglutination titrations. The effect of temperature on the chicken (F)-guinea pig (G) RBC agglutination ratio(7,8), determined with 1st passage fluids, was different for the 2 strains: the 1st amniotic passage NA strain showed a low F/G ratio at both 24.5 and 4.5°C, whereas the 1st allantoic passage RA strain showed a low ratio at 24.5 but not at 4.5°C. It is apparent that as early as the 2nd passage both the NA and RA strains showed high F/G ratios at both 24.5 and 4.5°C.

Antigenic similarity during early passages. Table V summarizes the results of complete cross-neutralization tests *in ovo* with 1st and later passage materials containing the NA or RA strain and corresponding immune sera. Several features require comment. In cross-neutralization tests with the NA strain, sera were used from roosters which had received 2 intravenous injections of virus. After only 1 injection of virus, the antiserum against the 1st passage amniotic strain had a neutralizing titer of but 1:2. This probably was due to the low concentration of virus in the 1st passage amniotic fluid. Cross-neutralization tests with the RA strain and either rooster or rabbit antisera were carried out with serum obtained after but 1 intravenous injection of virus. It is noteworthy that in all cross-neutralization tests 1st passage NA or RA strain appeared to be neutralized by a smaller quantity of antibody than was required with the same strain at a later passage.

As has been shown previously(3,9), the antigenic relation between 2 strains can be expressed in a single figure. To obtain this value, it is necessary to determine the 2 heterologous serum titer ratios from the results of a complete cross-serological experiment. The geometric mean (R) of these 2 ratios may be taken as the best available indication of the extent of the antigenic relation between the strains. R values, computed in this manner, are shown for the data presented in Table V. The fact that each of the R values for both the NA and RA strains closely approximates 1.0 is strong evidence that no definite difference in antigenic composition occurred between the 1st and later embryo passages.

Discussion. The fact that no significant antigenic variation was demonstrable between influenza A virus strains at the 1st and in later passages in the chick embryo is of considerable interest because it provides support for the assumption that strains recovered and passed in the chick embryo are comparable in antigenic composition to those causing dis-

TABLE IV. Changes in Hemagglutinating Capacity of NA and RA Strains During Early Passages.

Virus strain	Embryo passage No.		Hemagglutination titers			
			Temperature			
			24.5°C		4.5°C	
			Erythrocytes			
	Amniotic sac	Allantoic sac	Guinea pig	Chicken	Guinea pig	Chicken
NA	1		64	8	64	16
	1	1	512	256	512	1024
	1	2	512	256	512	512
	1	4	512	256	512	512
	1	7	1024	512	512	1024
RA		1	128	32	128	256
		2	512	512	512	1024
		3	2048	1024	1024	4096
		5	2048	1024	1024	4096

TABLE V. Cross-Neutralization Tests with the NA and RA Strains and Corresponding Immune Sera after 1st and Later Embryo Passages.

Immune serum vs.	Virus strain				
	NA, 1	NA, 8	NA, 1	NA, 8	
	Serum neutralizing titer*		Serum titer ratio		R value†
NA, 1‡	*76*	16	*1*	1/4.7	1/1.58
NA, 8	1000	*523*	1.9	*1*	
	RA, 1	RA, 5	RA, 1	RA, 5	
RA, 1§	*28*	19	*1*	1/1.4	1.07
RA, 5	27	*16*	1.7	*1*	
RA, 1‖	*860*	364	*1*	1/2.3	1/1.08
RA, 5	1450	*726*	2	*1*	

* Each titer represents the geometric mean of 2 titration end points.

† Computed from the 2 heterologous serum titer ratios as follows: $R = \sqrt{r_1 \times r_2}$ (3).

‡ NA, 1 = Rooster serum, 46-day, vs. infected amniotic fl, 1st passage from throat washing.
NA, 8 = " " " " " allantoic 8th " in chick embryo.

§ RA, 1 = " " 26-day, " " " 1st " from throat washing.
RA, 5 = " " " " " " 5th " in chick embryo.

‖ RA, 1 = Rabbit " 21-day, " " " 1st " from throat washing.
RA, 5 = " " " " " " 5th " in chick embryo.

ease in man. It has been demonstrated previously(3,6,9-15) that strains of influenza viruses recovered from patients in different epidemics show evidence of antigenic variation of considerable degree even though maintained exclusively in the chick embryo. It appears now that such variation is not to be attributed to unpredictable factors leading to the selection of variant strains in the chick embryo but instead is due to factors operative during the transmission of influenza viruses in man.

In contrast to the lack of variation in respect to antigenic properties, the reactivity of influenza A virus relative to guinea pig and chicken erythrocytes undergoes a profound and rapid change on passage in the chick embryo as has been emphasized previously (7,8). This alteration may occur as early as the 2nd embryo passage and persists thereafter. In addition, the results of the neutralization tests raise the possibility that the ratio between antibody and virus at the end point may undergo a change as a result of serial passage in the chick embryo. On the average, after 5-8 passages, it was necessary to use approximately twice as much antibody to cause virus neutralization as was required with the same strains after but 1 passage in the chick embryo. Because of the extraordinarily steep slope of the neutralization line *in ovo*(5), it would have been necessary to use consistently about 50 times more later passage than 1st passage virus in order to obtain the ratio differences noted. It should be emphasized that the quantities of 1st and later passage virus used in each of the neutralization tests were closely similar.

Summary. Cross-neutralization experiments *in ovo* with 1st and later chick embryo passage strains of freshly recovered influenza A viruses showed no definite difference in their antigenic composition. However, the reactivity relative to guinea pig and chicken erythrocytes underwent a rapid and profound change between the 1st and 2nd embryo passages.

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Effect of Relaxin Upon Decidual Reactions in the Rat.* (19789)

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The most important physiological actions known for relaxin are displayed in effects upon connective tissue(1-5). The range of such effects has been broadened by recent observations(6) that relaxin preparations possess the property of inhibiting the extensive modification of stromal connective tissue which accompanies the development of a deciduoma in the rat uterus. Quantitative comparison of responses to a series of relaxin preparations of widely different specific activities, experiments with relaxin from the blood of pregnant rabbits, and studies of the effects of chemical modification of relaxin all indicate that inhibition of decidual growth in rats is a characteristic property of this hormone. The data also show that some relaxin preparations contain a substance, clearly distinguishable from relaxin (and from the decidual inhibitor), which enhances the action of progesterone in the development of deciduomata.

Experimental. Quantitative study of factors affecting decidual growth requires a rigidly standardized means of producing deciduomata of uniform size. We have routinely employed 100-day-old estrous rats, averaging 200 g in weight, which are made pseudopregnant by electrical stimulation of the uterine cervix. On the fifth day of pseudopregnancy, they are ovariectomized, and the right horn of the uterus traumatized by a needle scratch along its entire length. Daily injections of progesterone (1.5 mg in 0.1 ml sesame oil) are given for 3 days, and the animals autopsied on the fourth.

In some of the earlier experiments, gross comparison of the 2 horns served as a qualitative indication of the response. Subsequently, in order to obtain data which would be susceptible of quantitative comparison, the 2

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horns were separated at the cervix and weighed separately. It has been found that the increase in weight of the traumatized horn serves as a measure of the extent of reaction. In order to minimize the effects of individual variation, the data are expressed in terms of the percentage increase relative to the weight of the untraumatized horn.

Solutions of substances to be tested for inhibition of decidual development were prepared in physiological saline, the concentration being adjusted so that the total desired daily dose was contained in 1.5 ml. One-half ml was injected 3 times daily for 3 days following uterine traumatization.

Relaxin preparations. Eight different preparations of relaxin from sows' ovaries and one concentrate of pregnant rabbit serum were employed during the course of this investigation. Of the former, one was isolated from ovaries of non-pregnant animals (preparation 70-1), and another (67-1) from unselected ovaries, of which it was estimated that about 20% were from pregnant animals. For the others, ovaries of pregnant animals were used. The method of preparation was that described previously(7), with slight modifications in some instances. Preparations 66-4 and 66-5, which assayed 450 and 300 GPU/mg, respectively, were obtained by acetone refractionation of material of lower specific activity.

A sample of 'Releasin'‡ was also used. The absolute specific activity of this preparation was not known; assays in our own laboratory showed that the solution contained about 250 GPU/ml. Pregnant rabbit serum (PRS) was concentrated by the procedure of Albert and Money(8). The lyophilized product had an activity of 4 GPU/mg. To serve as an appropriate control, a sample of male rabbit serum (MRS) was concentrated similarly.

Results. Preliminary experiments indicated that the daily dose of relaxin required for qualitatively complete inhibition lay in the range 100-300 GPU. More complete and quantitative data (Table I) indicate that this preparation (59-7) has 2 different effects upon the development of deciduomata. At fairly high levels (1.0-1.6 mg/da) the effect is clearly inhibitory; lower doses, on the other hand, appear to enhance the action of progesterone.

TABLE I. Effect of Preparation 59-7 (90 GPU/mg) upon Decidual Development.

No. of animals	59-7 injected mg/day	59-7 injected GPU/day	% increase*
5‡	.0	0	4 ± 16†
13	.0	0	345 ± 65
3	.17	15	715 ± 185
6	.33	30	800 ± 92
3	.51	45	585 ± 246
3	.66	60	440 ± 110
3	1	90	110 ± 38
3	1.33	120	36 ± 14
3	1.66	150	63 ± 38

$$* \ \frac{[(\text{Wt traum. horn})-(\text{Wt control horn})]}{(\text{Wt of control horn})} \times 100$$

† Avg ± stand error.

‡ These animals received no progesterone.

Data relating to the effects of a series of relaxin preparations (Table II) show that the inhibition produced by ovarian extracts is proportional to the relaxin content, rather than to the amount of material injected, over a 100-fold range of specific activity. Of particular interest are the data for preparations obtained from ovaries of non-pregnant animals (70-1) and from unselected ovaries (67-1). Only small amounts of relaxin have been obtained from the ovaries of non-pregnant sows; material from this source should thus serve effectively as a control for comparison with similar extracts from the ovaries of pregnant animals. Both of the above preparations appear to enhance the action of progesterone when tested at 2 mg/day, whereas an extract from glands selected from pregnant sows (66-6) shows good inhibition at half this level. Although preparation 70-1 at 5 mg/day affords more pronounced inhibition than would be expected on the basis of its relaxin content (when compared with other, more active relaxin preparations), the more pronounced inhibition obtained with 67-1 is correlated with its greater relaxin content. A possible explanation of the result with 5 mg 70-1 will be advanced subsequently.

It was found that a preparation of pregnant rabbit serum (PRS) displayed qualitatively the same features with respect to decidual in-

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‡ Supplied through the courtesy of Dr. Robert L. Kroc, Chilcott Laboratories, Morris Plains, N. J.

TABLE II. Effects of Ovarian Extracts upon Decidual Development.

Preparation No.	Specific activity (GPU/mg)	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
70-1	5	2	10	3	505 ± 110
		5	25	4	210 ± 100
67-1	20	2	40	3	470 ± 190
		5	100	6	75 ± 85
62-2	70	1	70	5	103 ± 40
		1.55	110	6	67 ± 55
		2.1	150	5	18 ± 17
59-7	90	.33	30	6	800 ± 92
		1	90	3	110 ± 38
		1.66	150	3	63 ± 38
66-6	100	1	100	6	70 ± 18
66-4	450	.375	170	4	4 ± 18
Releasin*	?	—	100	3	34 ± 29

* Chilcott Laboratories, No. CH-31-95-SF.

TABLE III. Effects of PRS and MRS upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
PRS	7.5	30	3	465±210
	15	60	3	425± 51
	25	100	3	380±110
	37.5	150	3	188± 77
	50	200	4	64± 21
	75	300	3	156±145
MRS	52	0	4	306± 29

hibition as relaxin from sow ovaries (Table III). Both produce potentiation at low and inhibition at high dosage. Quantitatively, however, significant differences are noted. Considerably less potentiation is observed with PRS, and, within the inhibitory range, ovarian extracts possess approximately twice the inhibitory activity of the same amount (in GPU) of PRS. That the inhibition by PRS is quite likely due to its relaxin content is indicated by the failure to elicit a response with a similar preparation from the serum of male rabbits (MRS).

Since small amounts of estrogen will effectively inhibit decidual formation under the conditions of these experiments(9), it was necessary to eliminate the possibility that contamination with estrogen was responsible for the effects observed. Four of the crudest relaxin samples (including PRS) were assayed for estrogen content. At levels which produced good inhibition of decidual growth, no significant effects were observed upon either the weight or water content of the uteri of ovariectomized rats.

Investigation of the chemical properties of the decidual inhibitor indicate that they are similar to those which have been established for relaxin. Among the characteristic chemical properties of relaxin is the relative stability of its solutions toward heat. Thus, neutral solutions of relaxin may be exposed to 100° for 10-15 minutes without appreciable loss of activity(10). Comparison of the effects of heated[§] and unheated relaxin upon decidual reactions (Table IV) shows clearly that the decidual inhibitor shares with relaxin the property of heat stability. Quantitatively, the heated preparation appears to be approximately 5 times as effective as the native material. This may be most readily explained by assuming that the substance in relaxin preparations which enhances the action of progesterone is heat labile; its destruction permits the inhibition to become apparent at lower concentrations, the effects of which are masked in unheated samples.

It should be noted that heated samples of PRS are also inhibitory at lower concentrations, and that the quantitative discrepancy between the effects of relaxin and PRS (Tables I and III) disappears when the effects of heated samples are compared (Fig. 1).

Relaxin has been shown to be sensitive to the action of a variety of reducing agents(11)

[§] A heating period of 10 minutes was chosen as optimal on the basis of preliminary experiments.

TABLE IV. Effects of Native and Heated Relaxin upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day	No. of animals	Avg % increase
66-6, unheated	0	0	13	345 ± 65
	.20	20	5	485 ± 53
	.30	30	5	345 ± 210
	.40	40	5	420 ± 120
	.60	60	5	230 ± 73
	.80	80	5	95 ± 82
66-6, heated 10 min., 100°	.05	5	5	300 ± 150
	.10	10	5	225 ± 89
	.20	20	5	90 ± 62
	.40	40	6	42 ± 44
	.60	60	8	60 ± 50

and to prolonged exposure to acidified methanol, while neither deamination nor acetylation under relatively mild conditions results in appreciable loss of activity(10). Samples of relaxin were treated with sodium thioglycollate, anhydrous methanol containing 0.1 N HCl, nitrous acid, and acetic anhydride, and the reaction products tested for decidual-inhibiting activity (Table V).

No trace of either potentiation or inhibition survives treatment with thioglycollate. The effect of nitrous acid, however, appears to be qualitatively and quantitatively similar to that of heating, *i.e.*, the inhibitory property is enhanced rather than diminished by deamination. The fact that less inhibition is observed at the one milligram level is probably of no especial significance, being most likely due to experimental variation. The effect of treatment with acetic anhydride is also qualitatively analogous to that of heating, but quantitatively the analogy is less satisfactory. It is clear that the inhibition displayed by 1 mg of relaxin 66-6 daily is unaffected by this reagent. The average increase in decidual horn weight found with acetylated material at 20 GPU/da., although considerably less than that found for the unreacted sample, is greater

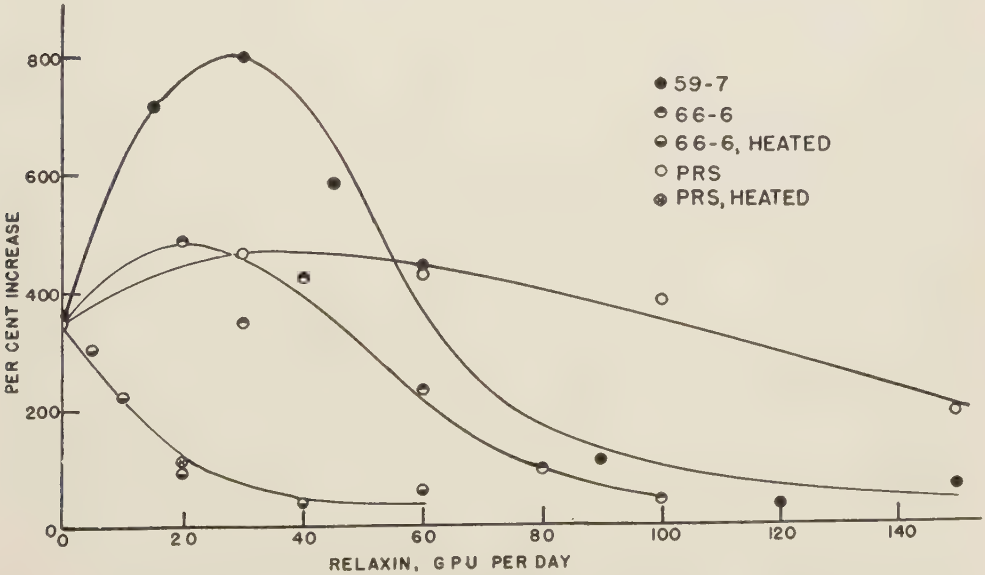

FIG. 1. Summary of effects of 2 unheated relaxin preparations (59-7 and 66-6), a sample of 66-6 which had been heated 10 min. at 100°, and PRS upon decidual development. One experiment with PRS heated 15 min. at 100° is included. Ordinate: % increase in wt of traumatized horn.

TABLE V. Effects of Chemically Modified Relaxin upon Decidual Development.

Preparation	Amt injected mg/day	Amt injected GPU/day[*]	No. of animals	Avg % increase
66-6	.20	20	5	485 ± 53
	1	100	6	70 ± 18
66-6, reduced[†]	7.50	750	2	325
66-6, deaminated[‡]	.20	20	5	58 ± 35
	1	100	5	95 ± 55
66-6, acetylated[§]	.20	20	5	203 ± 126
	1	100	5	46 ± 14
66-6, methylated[‖]	1	100	5	240 ± 96

[*] Calculated for the corresponding wt of unreacted material. [†] Treated with sodium thioglycollate, pH 7.1, for 15 hr. Reduction product isolated by 3 pptns. with acetone at pH 3. [‡] .1 M $NaNO_2$, pH 4, 0°, 30 min. [§] With 150-fold excess Ac_2O in NaOAc at 0°. [‖] Treated with MeOH containing .1 M HCl for 22 hr at 22°.

than that found with either heated or deaminated material at this level. It should be noted, however, that of the 5 animals used for this experiment, 2 displayed very considerable inhibition. The effects of acetic anhydride are assumed to be confined to destruction of the physiological activity of the potentiator. The variability observed may be due either to incomplete reaction with essential amino groups of the latter, or to some *in vivo* reactivation.

Methylation of relaxin results in 90% or greater inactivation as tested in the guinea pig. If it is assumed that the potentiating factor is completely destroyed by this reagent, retention of a small amount (10%) of decidual inhibiting activity would account for the results observed. It is also possible that the rat and the guinea pig differ somewhat in their ability to reactivate the methyl ester. An unequivocal choice between these 2 alternatives cannot be made on the basis of the available data.

Discussion. There can be no doubt that the phenomenon of decidual potentiation, especially prominent with small amounts of preparation 59-7, but observable in greater or lesser degree with most of the relaxin samples studied, is due to a substance other than relaxin and present as a contaminant. It may be distinguished from relaxin (and from the decidual inhibitor) by its sensitivity to heat, and by the fact that it contains essential amino groups, the loss of which, by either deamination or acetylation, reduces the potentiation observed. It is therefore to be expected that the magnitude of decidual potentiation would vary considerably from one relaxin preparation to another, as may indeed be observed to be the case (compare Tables I and IV). It was also to be anticipated that further purification of relaxin should lead to preparations essentially free of potentiator; these should be inhibitory at relatively low concentrations in the native state, and the effect of heating should be much less pronounced. We have been able to test this hypothesis with preparation 66-5, which assayed 300 GPU/mg (Table VI). From its occurrence in preparations which had been dialyzed during purification, and the properties described above, it may tentatively be concluded that the potentiator is a protein.

TABLE VI. Effect of Relaxin Preparation 66-5 (300 GPU/mg) upon Decidual Development.

Amt injected mg/day	Amt injected GPU/day	Period of heating	No. of animals	Avg % increase
.066	20	None	3	44 ± 30
.066	20	10 min.	3	18 ± 18
.330	100	None	2	31

While absolute identification of relaxin with the substance responsible for decidual inhibition in the rat must await the isolation of one or the other in indisputably pure form, all of the data so far obtained support the assumption that the two are the same. The close parallel between relaxin content and inhibitory activity of preparations from sows' ovaries which differ widely in specific activity affords good evidence for their identity, as does the comparison of the effects of PRS and ovarian relaxin. As has already been mentioned, the quantitative discrepancies between PRS and other relaxin preparations appear to be due to contamination by the potentiating factor. The presence of the latter in relatively small amounts in the ovaries of non-pregnant sows may possibly account for the unexpected activity of preparation 70-1 (Table II), since, when heated 10 minutes and tested at 2.0 mg/da., this preparation was only slightly inhibitory. Equally consistent with this assumption are the results of experiments with chemically modified relaxin.

Interaction between progesterone and relaxin shows marked species variation. Thus, while progesterone stimulates the formation of relaxin in the reproductive tract of the guinea pig(12), it (indirectly) prevents the action of relaxin upon the mouse public symphysis(13). Large amounts of relaxin are only partially effective as an inhibitor of decidual formation in mice(14), and the largest amounts tested (1000 GPU/day) were totally without effect upon decidual reactions in guinea pigs(15). In contrast to the situation in guinea pigs and mice, there is no evidence that relaxin, or an analogous substance, is involved in the adaptive mechanism of gestation in the rat; indeed, the administration of relaxin during pregnancy results in death and resorption of the fetuses.‖

The wide variations in the response of different species to relaxin serves to emphasize their common denominator. Relaxation of the pubic symphysis, deciduoma formation, and stromal changes in the mammary gland of the rat during lobulation, are all processes in which connective tissue changes predominate. It is possible, therefore, that the physiological process in which relaxin takes part may be a basic one common to certain kinds of connective tissue.

Summary. Preparations of relaxin from the ovaries of pregnant sows and from the blood of pregnant rabbits inhibit the development of a decidual reaction in the uterus of the castrate-pseudopregnant rat following traumatization and treatment with progesterone. Partially purified relaxin samples also contain a heat-labile factor which synergizes with progesterone in the production of deciduomata. On the basis of experiments with relaxin preparations covering a wide range of specific activities, and the effects of a variety of chemical reagents, it is concluded that the inhibitory property of such preparations is a specific response to relaxin.

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‖ The administration of 300 GPU of relaxin on each of the first 10 days following mating resulted in an average litter size of 3.4 (1.8 live young) as compared to the normal litter size of 10.2, in 5 animals.

Protective Effect of Cortisone on Induced Asthma in the Guinea Pig.*
(19790)

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Most of the working concepts in human allergy, such as the antigen-antibody reaction and the principles of sensitization and desensitization, have had their origin in observations on anaphylaxis in animals, particularly in the guinea pig. Nevertheless, the prevention of anaphylactic death as a method of studying the value of some therapeutic procedures proposed for allergy has been unreliable. Although fever, localized infections and other stresses frequently relieve asthma and other allergies in man, preliminary experiments showed that artificially induced fever or abscesses in sensitized guinea pigs failed to inhibit anaphylactic death. Then, too, although cortisone or adrenocorticotrophic hormone relieves the symptoms of asthma and many other allergic phenomena in the majority of sufferers, virtually all investigators agree that neither of these hormones is effective in protecting against anaphylactic shock.

This discrepancy between clinical allergy and anaphylactic shock in the guinea pig might be explained either on the basis of a total difference in mechanism or on a difference in degree, such as the intensity of the phenomenon of anaphylactic shock. Certainly the severity of the manifestation of human asthma is usually far removed from the violence of the reaction pertaining in the shocked animal. Indeed, in some instances where the allergic phenomenon was very acute and severe, approaching in intensity that of anaphylaxis in the guinea pig, [allergy to ACTH(1), acute reactions from aspirin(2)], cortisone was found incapable of inhibiting it. With these considerations in mind a procedure in the laboratory animal has been developed which would more closely approach in degree and kind the symptoms encountered in clinical allergy.

Methods. Male guinea pigs of about 350 g average weight were passively sensitized by the intravenous injection of 0.5 ml/kg of guinea pig antiovalbumin serum. These were divided into two groups. After 48 hours the control animals were subjected to an aerosol of a 0.2% solution of the homologous antigen in physiological saline introduced into the chamber at 5 lb pressure. The experimental group of animals was pretreated with 50 mg of cortisone (Cortone Acetate Merck) given as two divided doses, each injected into a gluteal muscle 18 hours before exposure to the antigen by aerosol. The animals were allowed to remain in the chamber and the time of exposure required to produce obvious symptoms of asthma (marked dyspnea and cough) was noted. The animals were removed from the chamber after 10 minutes if during this period no symptoms appeared.

Results and discussion. The results of 100 determinations for each group are shown in Table I. Each series consisted of approximately 50 guinea pigs which were subjected to an identical experiment after a 2 week rest period. The protective effect of cortisone is apparent from the fact that a large number of the hormone-treated animals tolerated the

TABLE I. Effect of Cortisone on Time Required to Produce Asthma by Aerosol in Sensitized Guinea Pigs.

No. of guinea pigs	Trial 1, mean in min.	Trial 2, mean in min.
	Control	
50	2.52	2.51
Mean of total	2.51	
σ^2	1.80	
	Cortisone treated	
50	5.94	4.43
Mean of total	5.19	
σ^2	21.75	
t	5.5	

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aerosol a longer time before the symptoms of asthma occurred. It has been calculated by the method of normal variables that a significant difference exists between the means of the times of exposure of the two groups. A value of $t = 5.5$ was obtained which indicates a significant difference with a confidence limit of better than 98%.

After a further 2 week rest period the control group of guinea pigs became the experimental, hormone-treated group, and vice versa. A significant difference, $t = 4.7$, was calculated for the means of these two groups.

The amounts of antiserum and the concentration of the antigen used were determined experimentally so that the appearance of asthma would neither occur too rapidly nor too slowly in the control group. It was found that the quantity of cortisone used and the time interval allowed to elapse before challenge were important criteria. The animals showed individual variation as to the rapidity with which asthma was produced, effectiveness of hormonal treatment, etc. This variation was not necessarily consistent in a repeated trial. None of the untreated, control guinea pigs failed to show typical symptoms of asthma, however, within the ten minute time limit which was arbitrarily chosen. Most of the cortisone-treated animals which survived a 10 minute exposure failed to show any of the more typical symptoms of asthma after they were out of the chamber; an increased respiratory rate was noticed in some of these animals.

Cortisone has been reported as being effective in suppressing experimental hypersensitivity of the Arthus type(3), in inhibiting the cardiovascular and renal lesions of hypersensitivity in rabbits(4), in preventing the anaphylactoid reaction in rats(5), in protecting mice (6) and rats(7) against anaphylaxis and guinea pigs against reversed passive anaphylaxis(8). The hormone has been found to be ineffective in specific antibody-antigen reactions resulting in anaphylaxis by the usual active and passive technics. The results herein reported indicate that cortisone does play a protective role in the anaphylactic type of hypersensitivity. Preliminary results indicate that ACTH also exerts a somewhat similar effect.

Summary. In a significant percentage of sensitized guinea pigs cortisone was found to be effective in inhibiting or delaying the symptoms of asthma induced by an aerosolized antigen.

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Determination of Quaternary Ammonium Compounds Including Acetylcholine, Tetraethylammonium, and Hexamethonium.*† (19791)

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The increasing pharmacological interest in quaternary ammonium compounds (QAC) stimulated our efforts to develop a method for their determination in biological media. The methyl orange method of Brodie(1,2) and the bromcresol purple method of Woods(3) are general reactions for organic bases, and

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therefore, limited in their usefulness because of formation of the dye complex with normally occurring organic bases extractable from the biological material. The major problem in finding a suitable method centered around the difficulty of quantitatively extracting the highly ionized QAC from biological media without contamination. The industrial procedures, which have been summarized by Dubois(4,5), and the precipitation methods, such as the reineckate salt method, lack the required sensitivity. The bromphenol blue method of Auerbach(6), however, seemed of greatest promise, and the method to be reported here is a modification of this. The great advantage of this bromphenol blue method is that it circumvents the necessity of first extracting the QAC from the test media. The reaction between the anionic bromphenol blue and the quaternary cation can be carried out, in the presence of alkali, in the original biological media and then the dye complex that is formed can be quantitatively extracted without interference from the unreacted dye or from any of the naturally occurring organic bases. The procedure as outlined below increased the sensitivity of Auerbach's method (6) more than tenfold. Smaller quantities of sample are used, but the increase in sensitivity is due mainly to the addition of phosphate-ion which was found to be essential for good color development. This reaction to form a complex salt in alkaline solution seems to be a property only of QAC and not of tertiary nitrogen compounds. Auerbach(6) tested 50 tertiary amines with negative results. Using the method reported here, negative results were also obtained with a number of the tertiary amines he tested as well as with other tertiary nitrogen compounds such as procaine.

The method to be reported has been divided into two main sections: 1) a general procedure and 2) a modified procedure.

General procedure. To date, it has been observed that the general procedure can be used for the determination of only those QAC in which at least one chain is 4 carbons or longer or $C_6H_5CH_2$—or modifications thereof; such compounds will be classified below as Group I. Some examples of Group I compounds that have been determined by the general procedure are: the methobromide of procaine and procaine amide; Banthine; the N-diethylmethyl ethanolamine bromide esters of 1-benzoylcyclopropanecarboxylic acid, of 2,2-diphenylpenten-4-oic, of α-phenoxycinnamic acid, and of 2-(*o*-chlorophenyl)-5,6-dihydro-4H pyran-3-carboxylic acid; the N-(2-hydroxyethyl)-N-methylpiperidinium bromide ester of 1-benzoylcyclopropanecarboxylic acid; the 1-hydroxy-2-(diethylmethylamine) cyclohexane bromide ester of 2,2-diphenylpenten-4-oic; and 4-benzoyl-4-carbethoxy-1,1-dimethylpiperidinium bromide.

Reagents. 1) *Dye solution. 40 mg of bromphenol blue*‡ *in 50 ml of a 30% K_2HPO_4 solution.* This reagent should be prepared each day. The presence of phosphate-ion was found essential for full color development, the color intensity increasing to a maximum at 0.2 *M* phosphate; excess phosphate-ion had no quenching effects. Therefore, the bromphenol blue reagent was made up in K_2HPO_4 solution in order to keep conditions constant and to avoid unnecessary dilution of the sample. The presence of carbonate, bromide, iodide, chloride, sulfate, acetate, citrate or oxalate ions did not affect the color development in any way. However, the presence of nitrate, even in the presence of phosphate, lessened the intensity of the color development with some compounds but not with others. 2) *Alkaline reagent. 0.8 M borate buffer, pH 9.0 or 20% Na_2CO_3.* The dye complexes of the majority of the compounds tested could be extracted by ethylene dichloride (EDC) from a solution made alkaline with borate buffer, pH 9.0. However, in the case of a few compounds it was necessary to use a stronger alkalinizing agent, 20% Na_2CO_3. 3) *Extracting Solvent. Washed EDC containing 1½% by volume of iso-amyl alcohol.* The EDC used must be washed each week. It was noted that the blue color complex faded very rapidly in EDC not washed this often, but remained stable for at least 1 hour in freshly prepared EDC. The EDC was washed with ⅕ vol. I *N* NaOH, then ⅕ vol. 1 *N* HCl, followed by 3 washes with distilled water. Iso-amyl alcohol, washed similarly, was added to the washed EDC to minimize any possibility of adsorption of the complex from the solvent onto the glass surface. EDC is the solvent of choice since it does not extract any of the unreacted dye, and reagent blanks read zero against EDC.

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‡ National Aniline Division, Allied Chemical and Dye Corp., Lot No. 14046.

Procedure. To a sample, not larger than 2 ml, containing the QAC, 1 ml of appropriate alkali, 1 ml of bromphenol blue reagent and 10 ml of the EDC were added. This was shaken mechanically for 10-15 minutes in a 50 ml glass-stoppered Erlenmeyer flask. The contents of the flask were then centrifuged for 3-5 minutes, at 1500 RPM. The upper layer was aspirated off completely and the lower layer (EDC) was decanted into a dry cuvette and read in a spectrophotometer at 600 mμ against an EDC blank. The samples can be read immediately but the color is stable for at least 1 hour. No blank reading is given by the reagents or normal control media.

Standardization. Standard curves were made for each compound tested. A plot of optical density against the concentration of the QAC yielded straight lines from 2.5 to 50 μg per sample. The lower limit of sensitivity varied but in most cases was around 2-3 μg per sample. With this general procedure it was not necessary to run standards with each determination unless the dye lot was changed. *Recovery.* The recovery of known quantities of a number of compounds from serum or plasma diluted 1-10, buffer solutions, nerve homogenates, and from dilute homogenates of liver, kidney, and heart was 100 ± 2%. When the amount of the QAC in serum or plasma was too small to allow for dilution, it was necessary to make the changes outlined in the next section to obtain complete recovery. The general procedure had to be altered to effect recovery from urine only when the QAC was present in amounts too small to permit adequate dilution of the urine; these changes will be discussed under modified procedures.

Procedure for Group I in undiluted serum (or plasma). With undiluted serum it was necessary to precipitate the proteins before extraction of the QAC. All precipitants tried interfered with the final color development except HPO_3. Therefore the serum was precipitated with an equal volume of 6% HPO_3, allowed to stand for about 10 minutes, and then centrifuged for 15-20 minutes at 2000 RPM. To 3-4 ml of the clear supernatant, 1.5 g Na_2CO_3 and 0.2 g K_2HPO_4 were added, followed by 1 ml of the bromphenol blue reagent and 10 ml of EDC containing, in this case, 3% iso-amyl alcohol. The rest of the procedure was the same as outlined in the general method. The lower limit of sensitivity for the compounds thus determined was about 2 μg per ml of original sample. Standard curves were run for each compound in control serum precipitated with HPO_3. It was found necessary to vary the aliquot of supernatant and/or the amount and kind of alkaline salt used in the determination of small quantities of certain compounds in undiluted serum. For example, $Na_2B_4O_7 \cdot 10\ H_2O$ (1.5 g) was substituted for Na_2CO_3 in the determination of Banthine. Therefore, this procedure is suggested as a guide in the development of a method suitable for the particular compound to be determined. A number of Group I compounds have been recovered from undiluted serum using the above procedure, but difficulties have been encountered in a few instances. Efforts are being continued to develop sensitive procedures for these exceptions.

Modified procedure. The general procedure could not be used for the determination of acetylcholine (ACh) and hexamethonium (C_6), and was found to be 10 times less sensitive for *d*-tubocurarine and neostigmine than for other compounds in Group I. However, for these compounds as well as for tetraethylammonium (TEA), this general procedure has been adapted for use in certain media as shown in Table I. With the exception of the changes indicated in Table I the remainder of the procedure was the same as outlined above for the general method. It is essential to keep the aqueous phase at a minimum for ACh and TEA.

Procedure for undiluted urine. When the amount of QAC in the urine was too small to permit dilution, modifications of the procedure had to be devised. The recovery from undiluted urine varied with specific gravity of the urine tested and with the particular compound determined. All attempts to remove

TABLE I. Examples of Modified Procedures Suitable for Compounds Named in the Media Listed.

Compound	Medium from which extracted	Max aliquot of sample, ml	Alkaline salt used, g	Dye reagent, ml	Total aqueous phase, ml*	% iso-amyl alcohol in EDC	Limit of sensitivity, µg/sample
C_6	Urine (undiluted)	1	1.2 K_2HPO_4 .6 Na_2CO_3	1	5	1.5	2.5
ACh†‡	Ringer's sol. & nerve tiss.	.5	.3 K_2HPO_4 .3 Na_2CO_3	.5	1	4	1
TEA	Ringer's solution	.5–1	.3 K_2HPO_4 .3 Na_2CO_3	.5	1–1.5	4	1.5
	Urine (undiluted)	.5	.4 Na_2CO_3	.5	1	4	2.5
	Serum (diluted 1-10)	1	.8 Na_2CO_3	.5	1.5	4	2.5
Neostigmine	Ringer's solution	1	.4 Na_2CO_3	.5	1.5	4	2.5
	Urine (undiluted)	1	.8 Na_2CO_3	.5	1.5	4	2.5
d-Tubocurarine	Ringer's solution	1	.4 Na_2CO_3	.5	1.5	4	2.5
	Urine (undiluted)	1	.8 Na_2CO_3	.5	1.5	4	2.5

* Where aqueous phase is greater than sum of sample and dye, distilled water was added.
† Period of shaking limited to 10 min. ‡ Lower limit of sensitivity for choline is 15 µg/sample.

the interfering components of urine, either by precipitation, adsorption or extraction, so far, have proved ineffective. These urine components did not interfere by producing a urine blank, but rather prevented the complete recovery of the complex formed between the bromphenol blue and the QAC. For undiluted urine the method was modified in a manner similar to that above, *i.e.*, solid alkaline salts were used. Moreover, in some cases, the addition of 2-3 ml of water as well, improved the degree of recovery. The density of the aqueous phase should be greater than that of the EDC phase. Standards were always prepared in a control urine to insure accuracy. A few typical examples of the modifications used in the procedures for undiluted urine are outlined in Table I, and these may serve as a guide for the development of procedures for other compounds of interest.

Discussion. This method is presented as a general procedure for the estimation of QAC but there is no claim that it is universal. In addition to the compounds already mentioned at least 30 other Group I compounds were determined by this general method. But, as in the case of *d*-tubocurarine, neostigmine, and acetylcholine, there may be other compounds that fall into Group I and yet cannot be satisfactorily determined by the general procedure. The degree of recovery of a compound has also varied from one medium to another; *e.g.*, it is possible to determine small amounts of C_6 in urine with the procedure in Table I, but, as yet, no sensitive procedure has been developed for its recovery from undiluted serum. The reverse is true of Banthine which can be recovered from serum in low amounts but from urine only when present in much larger concentrations. Also, the degree of recovery of some compounds from different samples of urine showed variation. Despite these limitations, the method has been quite useful, and even though, at present, procedures have not been completed for each compound in each type of medium, it is hoped that a report at this time will prove of value.

However, certain generalities seem to be valid, namely that: 1) of the compounds tested so far, only those of the general structure which includes at least one chain of 4 carbons or longer, or $C_6H_5CH_2$—or modifications thereof, can be determined by the general method; 2) under alkaline conditions, no tertiary nitrogen compound has as yet been found which can be determined by the method; 3) there is no blank for the reagents, or normally, for control media.

Moreover, the general method can be used to distinguish Group I compounds from compounds which do not fall into this category. Thus, the general method has proved valuable in following the metabolism in serum (diluted 1-10) of some of the esters mentioned above (7). The hydrolysis of the ester could be determined without interference from its quaternary metabolite since only those esters were selected for this study which did not form metabolites belonging to Group I.

Summary. A general method is presented for the determination in buffer solutions, nerve homogenates, dilute serum, plasma, and urine of quaternary ammonium compounds in which at least one chain is 4 carbons or longer or $C_6H_5CH_2$—, or modifications thereof. The lower limit of sensitivity, using this procedure, is about 2-3 μg per sample. Adaptations of this method allow for the estimation of these compounds in undiluted serum or plasma. Acetylcholine, hexamethonium, tetraethylammonium, neostigmine and *d*-tubocurarine can be determined in certain media, in concentrations as low as 1-2.5 μg per sample, by means of modifications of the general procedure.

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Anaphylactic Shock in the Pertussis Vaccinated Mouse. II. Diphtheria and Tetanus Toxoids as Antigens.[*] (19792)

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The observation(1,2) that mice immunized by a mixture of *H. pertussis* organisms and horse serum become markedly anaphylactogenic to a challenging dose of the serum led us to study other antigens. The effect of pertussis vaccination on increasing the sensitizing capacity of diphtheria and tetanus toxoids was particularly interesting because of reported accidents, such as the clinical report(3) of fatal anaphylaxis following reinjection of a pertussis-diphtheria toxoid vaccine.

Methods. The technic utilized was that previously described(1,2). Female, white mice were inoculated intraperitoneally with 0.5 ml of a mixture containing about 8,750 million phase I organisms of *H. pertussis* vaccine, Sharp and Dohme, and 0.1 ml of either diphtheria or tetanus toxoid. The toxoids incorporated in this mixture were as follows: diphtheria toxoid, fluid; diphtheria toxoid, alum precipitated; tetanus toxoid, fluid; tetanus toxoid, alum precipitated.[†] Control groups of mice receiving only the diphtheria toxoid were injected at the same time as the experimental group. Fifteen days after immunization the mice were challenged by the intravenous injection of 0.1 ml of the respective fluid toxoid. The mortality rate was determined as the number of animals dead after 24 hours/number of animals injected.

Results and discussion. The results are tabulated in Table I. It can be seen that *H. pertussis* vaccine enhances the anaphylactogenicity of fluid diphtheria toxoid but apparently has no effect on tetanus toxoid. It was not deemed necessary to challenge the tetanus toxoid control groups since it had already been observed that the toxoid com-

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[*] This investigation was supported, in part, by a research grant from Sharp and Dohme, Inc., West Point, Pa.

[†] The toxoids used in this study were Diphtheria Toxoid, Illinois Department of Public Health; Diphtheria Toxoid, Alum Precipitated, Lilly; Tetanus Toxoid Fluid, Lilly; and Tetanus Toxoid, Alum Precipitated (Refined), Wyeth.

TABLE I. Enhancing Effect of *H. pertussis* Vaccine on Anaphylactogenicity of Various Toxoids; Mortality Rate = No. of Animals Dead after 24 Hr/No. of Animals Challenged.

	Toxoid alone	%	Toxoid combined with pertussis	%
Mortality in mice challenged with toxoid intrav. 15 days after intraper. inj. of				
Diphtheria, fluid	2/40	5	25/33	76
" , alum precipitated	12/38	31	14/26	54
Tetanus, fluid	—	—	4/38	11
" , alum precipitated	—	—	1/35	3
Mortality in mice challenged with toxoid intrav. 10 days after intraper. inj. of				
Diphtheria, alum precipitated	2/18	11	25/64	39
Tetanus, fluid	—	—	2/21	10
" , alum precipitated	—	—	0/10	0

bined with pertussis vaccine caused no increase in mortality rate. The difference in behavior between tetanus and diphtheria toxoid is perhaps a reflection of a possible difference in their antigenic capacity in the mouse.

The high mortality of the control group receiving alum precipitated diphtheria toxoid may be the combined effect of the adjuvant action of the alum acting over the 14-day period. It has been observed(5) that even after 10 days a high mortality rate results following antigenic challenge of a series of mice sensitized with a mixture of horse serum and vaccine. This shorter incubation period was utilized in another series of experiments with the tetanus toxoids and diphtheria toxoid, alum precipitated. One group of mice was injected with 0.5 ml of a solution containing 8,750 million *H. pertussis* organisms and ½ a human immunizing dose of toxoid. The control group received the toxoid alone. The animals were challenged after 10 days using 0.1 ml of the tetanus or diphtheria toxoid, fluid, given intravenously. The results also appear in Table I. The enhancement of the anaphylactogenicity of diphtheria toxoid, alum precipitated, by pertussis is more strikingly apparent. On the other hand, alteration of the size of the dose and time interval before challenge had no effect on the anaphylactogenicity of the tetanus toxoids.

It should be noted that the mortality rate in these studies is considered as the number of animals dead after 24 hours. Our observations have subsequently shown that the mortality rate can be recorded after 4 hours. Only rarely does an animal succumb after this period of time. We have made no attempt in these studies to record the appearance of signs of anaphylaxis other than the fatalities, such as described by Nelson, Fox, and Freeman(4).

Summary. 1. Mice immunized by an intraperitoneal injection of a mixture of *H. pertussis* and diphtheria toxoid become markedly anaphylactogenic to a challenging dose of toxoid. 2. Under the conditions of the experiments described pertussis vaccine has no enhancing effect on the anaphylactogenicity of tetanus toxoids.

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Histamine Shock and Vitamins. (19793)

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Though there has been much speculation on the antiallergic effect of various vitamins, only recently have experiments been reported which indicate that vitamins possess antianaphylactic and antihistaminic effects. For instance Traina(1) working with guinea pigs found that vit. B_{12} gives protection against anaphylactic shock and against twice the LD_{50} of histamine. Karady and Browne(2) showed that the previous administration of histaminase gives protection against anaphylactic shock, and suggested that vit. B_{12} may activate this enzyme. On the other hand, Guidolin and Ferri(3) in more extensive experiments reached the opposite conclusion, that vit. B_{12} does not protect against anaphylactic shock. Likewise Simon(4), in a clinical investigation, failed to find any antiallergic effect of B_{12}. Various authors have tried to relate vit. C to allergy. It has been recommended in high doses for the symptomatic treatment of asthma and other allergic syndromes. On the contrary, Friedell *et al.*(5), Holtz(6), and Wirtschafter and Widmann(7) reported that ascorbic acid contributes to the transformation of histidine into histamine both *in vitro* and *in vivo*. Also, it has been studied in relation to stress, the general adaptation syndrome, and the pharmacology of ACTH and cortisone. Finally, Saylor(8), Black(9), and Clarck and Mackay(10) reported various cases of allergic syndromes, especially urticaria, treated with vit. P and K. These contradictory results have led us to undertake investigations of the possible antihistaminic action of the principal vitamins.

Procedure and results. Our experiments were arranged as follows: a) the vitamins were given previous to the histamine shock, in order to measure the possible protective action; b) determinations of the effect of histamine on blood pressure were made before and after administration of the vitamins; c) the antihistaminic effect of the vitamins on the isolated guinea pig's ileum was studied.

a. *Histamine shock.* Histamine was given to guinea pigs as an aerosol in an "asthma chamber." The animals weighed 200 to 250 g. An aqueous solution of histamine (2 or 5 parts in 1,000) was dispersed with an air pressure of 300 mm Hg. The animals were first subjected to the histamine spray without prior treatment and the time from the start of histamine inhalation to loss of upright posture (histamine time, H_t) was measured; at H_t, the animals were taken out of the chamber. In these pretests, H_t was 2 to 3 minutes with a histamine concentration of 2:1,000 and 1½ to 2 minutes with a 5:1,000 concentration. After the pretests, the guinea pigs were divided into groups of greatest possible homogeneity as to H_t. After 5 days, the vitamin was administered subcutaneously 20 minutes before exposure to the aerosol. The results are given in Table I, where n is the number of guinea pigs, H_t the mean histamine time determined in the pretest, MH_t ("modified histamine time") the mean histamine time in the vitamin experiments, and P the probability value indicating the statistical significance of the difference between H_t and MH_t.

According to the data in Table I, neither 15 nor 30 μg/kg of vit. B_{12} gave any protection against histamine shock produced by exposure to histamine aerosol. Results were equally negative with 50 and 100 mg/kg of vit. C and also with vit. B_1, B complex and K.

b. *Vasodepressor action of histamine.* The fall of blood pressure produced by intravenous injection of histamine before and one minute after intravenous injection of vit. B_{12} was measured both in cats and in guinea pigs. The details are surveyed in Table II.

In the same arrangement, the effects of still higher intravenous histamine doses, sublethal and lethal, were studied in guinea pigs; blood pressure and respiration were simultaneously registered. The toxic symptoms—hypertensive phase, followed by a hypotensive phase after a lethal dose; bronchospasm; tachypnea followed by bradypnea—were qualitatively

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TABLE I.

Substance	Dose	n	Histamine spray conc.		H_t	MH_t	P
Vit. B_1*	10 mg/kg	10	5:	1.000	2′10″	1′55″	.5
B_{12}†	15 μg/kg	5	5:		1′30″	1′35″	.8
B_{12}	30	7	2:		3′30″	3′50″	>.1
C‡	50 mg/kg	10	2:		2′00″	2′25″	.5
C	100	5	5:		1′20″	1′35″	.7
K§	10	10	5:		1′25″	1′35″	.8
B complex‖	1 cc/kg	5	2:		2′05″	2′15″	.7

* Thiamin chloride, 1% sol. † 1.5:100000. ‡ 5% sol. § Menadion, 1% sol.

‖ Thiamin chloride 3 mg, riboflavin 1 mg, pyridoxin 1 mg, niacinamide 10 mg, calcium pantothenate 2 mg/1 cc aqueous sol.

TABLE II.

	Dose					
Species	Vit. B_{12}, μg/kg	Histamine, μg/kg	n	$-\Delta p_1$	$-\Delta p_2$	P
Cat	10	.25	10	18	18	.9
	15	.50	9	23	22	.8
Guinea pig	10	.50	10	10	11	.9
	20	1	10	15	15	.9

$-\Delta p_1$ = Fall of blood pressure (mm Hg) due to inj. of histamine, before administration of the vit.

$-\Delta p_2$ = Idem, after administration of the vit.

TABLE III.

Vitamin	Dose per 50 cc bath	n	h_{c1} (mm)	h_{c2} (mm)	P
B_{12}	1 mg	9	22	23	.7
	15	10	21	22	.8
	30	9	21	20	.7
C	1 μg	8	23	22	.9
	10	10	20.5	23	.7
	100	9	22	21	.8

h_{c1} = Ht of contraction due to histamine before addition of vitamin.

h_{c2} = Idem, after addition of vitamin.

and quantitatively no different in untreated animals and in those pretreated 1 to 15 minutes beforehand with 15 or 30 μg/kg of vit. B_{12}.

c. *Histamine action on excised guinea pig gut.* The preventive action of varied doses of vit. B_{12} and C against histamine spasm of the isolated gut was studied with ileum strips suspended in 50 cc Ringer solution. Before and one minute after addition of the vitamin dose, 1 μg of histamine was added to the bath fluid. After each histamine test, the intestine was washed with 100 cc Ringer solution. The mean value of the height of the histamine contraction (in mm) was determined in approximately 10 experiments in each group. Mean values were practically the same in the presence and in the absence of the vitamins, as shown in Table III. Thus, on this test object vit. B_{12} and C proved also to be devoid of antihistaminic action. Large doses of both vitamins (100 mg of C and 15 μg of B_{12}) produced marked relaxation, but not even in the relaxed state was the sensitivity of the intestine to histamine decreased.

Discussion. In the work here reported, vit. B_{12} failed to give any protection against the respiratory, the vasodepressor, the general toxic and the lethal actions of histamine or against the histamine-induced spasms of the isolated intestine. Against some of these histamine actions, vit. B_1, C, K, and B complex were also tried with the same negative result. The present experiments differ from those of Traina(1), who came to the opposite conclusions, in that we employed a greater number of test objects, conducted a larger number of experiments and extended the experiments into a higher dosage range of the vitamins; we also avoided the intracardial administration of vitamin and histamine, exclusively employed in Traina's experiments.

Summary. 1. Even in high doses, vit. B_1, B_{12}, C, K, and B complex did not protect guinea pigs against histamine shock produced by inhalatory administration of histamine aerosol. 2. Vit. B_{12} and C did not exhibit any antihistaminic action in assays of blood pressure in cats and guinea pigs. 3. Vit. B_{12} and C did not show any antihistaminic action in assays on the isolated guinea pig ileum; in high doses both vitamins caused the intestinal

strip to relax, but without decrease of its histamine sensitivity.

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Activity of Citrovorum Factor for the Chick.* (19794)

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Sauberlich and Baumann(1) reported the existence of a substance which was closely related to folic acid and which was necessary for the growth of *Leuconostoc citrovorum.* The isolation of citrovorum factor (CF) in crystalline form from horse liver was reported by Keresztesy and Silverman(2). This substance was found to be approximately twice as active for *L. citrovorum* as the synthetic compound, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (leucovorin(3), folinic acid-SF (4)). The biological activity of the natural crystalline product in folic acid deficient animals has not been reported thus far. Various reports have indicated that synthetic compounds with CF activity prepared from folic acid are at least partially active in folic acid deficient chicks. Hill and Briggs(5) reported that a synthetic form of folinic acid which differed from folinic acid-SF[†] was as effective as pteroylglutamic acid (PGA) when given in the diet. Broquist *et al.*(6) recently found that leucovorin was 50% as active as PGA when injected. Orally administered leucovorin, however, had considerably lower activity. The authors suggested that leucovorin might be a mixture of diastereoisomers in order to account for its having only one-half as much activity as PGA when injected. Recently, Sauberlich(7) compared the activity for the chick of PGA with a concentrate of CF and concluded that they had the same activity when incorporated in the diet.

Because of the differences in the reported activities of the various products and because of the recent availability of crystalline natural CF, it became of interest to determine the comparative efficacy of crystalline CF, folinic acid-SF, leucovorin, and PGA for the chick.

Experimental. New Hampshire female chicks from a commercial hatchery were equilibrated according to weight in groups of 6 and placed on experiment when one day old. They were raised in electrically heated brooders with wire screen floors. Feed and water were supplied *ad libitum* and the experiments were terminated at the end of 4 weeks. The basal diet, which contained no added folic acid, consisted of the following in g/kg: Cerelose (glucose) 615, vitamin-free casein 200, gelatin 80, DL-methionine 3, corn oil 40, Chick Salts A 60,[‡] and choline chloride 2.

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[*] The sample of leucovorin was supplied by Lederle Laboratories, Pearl River, N. Y., and the sample of folinic acid-SF by Dr. William Shive, University of Texas, and Eli Lilly and Co., Indianapolis.

[†] A personal communication from Dr. William Shive informs us that the folinic acid used by Hill and Briggs(5) was a purified amorphous synthetic sample which differed from folinic acid-SF, and did not contain any free or conjugated PGA.

[‡] Sixty g of Chick salts A contributed the following in g/kg of diet: $CaCO_3$ 15, K_2HPO_4 9, Na_2HPO_4 7.3, $Ca_3(PO_4)_2$ 14, NaCl 8.8, $MgSO_4 \cdot 7H_2O$ 5, ferric citrate (16.7% Fe) 0.4, $MnSO_4 \cdot 4H_2O$ 0.42, KI 0.04, $ZnCO_3$ 0.02 and $CuSO_4 \cdot 5H_2O$ 0.02. All salts were U.S.P. grade or purer.

TABLE I. Effect of Pteroylglutamic Acid and CF Active Compounds on Chick Growth.

		Avg wt in g at 4 wk, S.E.,* and No. of chicks†			
Group	Supplement/kg basal diet, mg	Series 6	Series 9	Series 16 (with aureomycin)‡	Series 24 (with aureomycin)‡
1	0	205 ± 9.5 (12)	254 ± 21 (6)	148 ± 18.4 (5)	148 ± 25.2 (5)
	Pteroylglutamic acid				
2	.1	334 ± 25.8 (6)	323 ± 29 (5)	222 ± 21.8 (6)	262 ± 18.7 (6)
3	.2	331 ± 13.6 (6)	380 ± 23.6 (6)	—	—
4	.4	376 ± 22.3 (6)	—	—	—
5	3	371 ± 38.4 (12)	411 ± 15.3 (6)	368 ± 15 (6)	345 ± 18.2 (6)
	Leucovorin				
6	.1	—	—	136 ± 9 (6)	—
7	.2	231 ± 22.6 (6)	260 ± 24.6 (6)	—	—
8	.4	262 ± 27.3 (6)	—	—	—
9	3	—	428 ± 32.8 (6)	—	—
	Citrovorum factor				
10	.1	224 ± 23.4 (5)	—	150 ± 14.3 (6)	157 ± 14.3 (6)
11	.2	248 ± 5.5 (5)	—	—	—
	Folinic acid-SF				
12	.25	238 ± 26.3 (6)	—	—	—
13	.5	232 ± 35 (6)	—	—	—
14	3.2	—	341 ± 26.1 (6)	—	—
	Pteroylglutamic acid inj.				
15	.1§	—	284 ± 12.1 (6)	264 ± 12.6 (5)	248 ± 8.2 (6)
	Leucovorin inj.				
16	.1§	—	—	190 ± 16.5 (6)	—
17	.2§	—	339 ± 13.6 (6)	—	—
	Citrovorum factor inj.				
18	.1§	—	373 ± 22.8 (6)	251 ± 15.3 (6)	232 ± 18.2 (6)

* S.E. determined by method of Mantel(8).

† No. of chicks remaining at end of experiment given in parentheses (6 chicks started per group). Groups 1 and 5 in Series 6 are averages of 2 groups.

‡ Each group in Series 16 and 24 was given 50 mg of aureomycin/kg of diet.

§ Injections made 3 times per week. All levels given were in proportion to amount of folic acid consumed by Group 2 during preceding period (see text).

The following vitamins were used (in mg/kg): thiamine hydrochloride 8, riboflavin 8, Ca pantothenate 20, nicotinic acid 100, pyridoxine hydrochloride 8, D-biotin 0.3, vit. B_{12} 0.02, vit. A acetate 3, vit. D_3 0.02, alpha-tocopherol acetate 10, and 2-methyl-1,4-naphthoquinone 1. The B vitamins were added to the diet by the use of dry premixes with Cerelose as the carrier. The fat soluble vitamins were added from stock solutions with corn oil as the carrier. Aureomycin was added to each diet in series 16 and 24 at a level of 50 mg/kg in order to enhance the severity of the deficiency (unpublished data). In series 6 and 9 the barium salt of the natural CF was used, whereas the calcium salt was used in series 16 and 24. In all instances the calcium salt of leucovorin and the free form of folinic acid-SF were used. All of these compounds as well as PGA were made up in slightly alkaline aqueous solution for subcutaneous injection and kept under refrigeration. The amounts administered are given in the table as the free forms. The amount of each compound injected corresponded to the amount of PGA consumed between injection periods by the group fed 0.1 mg PGA/kg of diet. Injections were given subcutaneously 3 times weekly.

Results. The average 4 week weights of the various groups from 4 different series are given in Table I. The addition of PGA to the basal diet resulted in typical growth responses. The feeding of CF, leucovorin, or folinic acid-SF at low levels resulted in substantially less growth than corresponding levels of PGA. Relatively high amounts of leucovorin and folinic acid-SF, however, caused good growth. Large amounts of CF were not used because of lack of sufficient amount of this compound.

CF and leucovorin were considerably more active when injected than when fed. Natural CF when injected appeared to be as active as PGA and somewhat more active than leucovorin (see series 9 and 16). A study of the significance of these differences by Fisher's "t" test (Snedecor) showed that these differences between injected CF and leucovorin were significant in series 16.

In general, the quality of feathers obtained by feeding or injecting the various CF active compounds could be correlated directly with the growth response.

Discussion. From the results obtained it appears that when crystalline CF is injected it is as active as PGA in the stimulation of the growth of chicks. Leucovorin is not as active as CF when injected. Broquist *et al.*(6) have previously reported that injected leucovorin was approximately half as active as PGA. This finding is substantiated by your data.

The low activity of CF, leucovorin, and folinic acid-SF when given in the diet may be due to possible modification of these compounds under the acid conditions which exist in the gizzard of the chick. Under such conditions it is known that alteration of the CF molecule may occur(9-11), whereby it might become inactive or unavailable to the chick. Further studies are necessary to explain the reported discrepancies in activity of the various compounds. Our results do not preclude the possibility that CF active compounds may be found in the future with chick activity when fed. The reports of Hill and Briggs(5) and Sauberlich(7) suggest that such a possibility exists.

It has been reported by Frost and Dann (12) that PGA was approximately twice as active when given by injection to chicks as when given orally. Our evidence indicates that the route of administration did not affect the activity of PGA although the method and time of injection may account for differences in this report.

Summary. 1. Crystalline citrovorum factor obtained from horse liver was approximately as active when injected as pteroylglutamic acid in counteracting folic acid deficiency in the chick. Under the same conditions citrovorum factor appeared to be somewhat more active than leucovorin. 2. Citrovorum factor, leucovorin, and folinic acid-SF when given in the diet were substantially less active than pteroylglutamic acid.

We wish to thank Mrs. Ligia Ortiz for helpful technical assistance in these studies.

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Failure of "Reticulose" to Reduce Radiation Lethality in Mice and Rats.* (19795)

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In recent reports it was stated that a lipoprotein-nucleic acid complex, Reticulose,† given to mice increased their leucocyte counts and resistance to infection(1), and given to rabbits after whole-body X-irradiation inhibited leukopenia(2). No experiments showing its effect on survival after irradiation were reported. Because of our particular interest in the role of infection in post-irradiation injury, we conducted experiments on mice (with and without added streptomycin) and rats to test this point.

Procedures. Male mice of an inbred NIH strain were used, maintained and irradiated as previously described(3), and caged singly beginning one week prior to irradiation. They were irradiated at the age of approximately 7 weeks and distributed among treatment groups in such a way that litter-mates and radiation groups were represented as equally as possible. Radiation factors were 200 KV, 20 ma., 0.25 mm Cu and 0.51 mm. Al added filtration, 50 cm distance to target, and radiation dose in air 625 r. The rats used were males of the Holtzman strain received at weaning and irradiated 3 weeks later at the age of approximately 7 weeks. They were caged singly from one week prior to irradiation, and were irradiated 12 at a time, factors being the same as with mice except that the distance to the target was 75 cm and radiation dose 570 r. Mice were given daily subcutaneous injections of 0.2 ml. Reticulose diluted 1:1 with sterile saline at the time of injection, beginning the day following irradiation and continued for 16 days. Streptomycin sulfate, 5% in saline, was used as the diluent instead of saline in one group, and its control was given 0.2 ml of 2.5% streptomycin sulfate so that each of these groups received 5 mg per mouse per day for 16 days. Rats were given 1.0 ml of undiluted Reticulose daily for 7 days beginning the day after irradiation. Sterile precautions were observed in making the injections, and only freshly opened ampules of Reticulose were used. Leucocyte counts were made‡ only on the mice, separate groups being set aside for this purpose. Tail blood was used and no mouse was bled oftener than once a week.

Results. In Table I it is seen that Reticulose administration affected neither the percent surviving nor the time of death in mice given 625 r or rats given 570 r. Although streptomycin treatment significantly reduced the mortality in mice (see also Miller *et al.*) (4), Reticulose did not influence this effect in either direction. The uniformity of the results is well within the limits of variability to be expected from chance differences alone.

Table II shows that Reticulose administration did not significantly affect leucocyte counts of mice with or without radiation, with or without streptomycin treatment. Furthermore, while streptomycin treatment resulted in a significant reduction (of about 1/3) in the number of mice showing positive heart blood cultures at autopsy, Reticulose, with or without streptomycin, had no effect on the incidence of infection, which averaged 33 to 34% of the mice irradiated, with or without Reticulose.

Discussion. In the data of Berry and Mitchell(1) concerning resistance of Reticulose-injected mice to infection, on the day when the cumulative survival curves show the widest separation, the value for χ^2 (3.6) indicates a probability somewhat greater than one in 20 that the difference was due to chance alone, and this difference was nullified a few days later. Concerning the effect of Reticulose on leucocyte counts (mean of 7 = 17,350

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† 'Reticulose' was generously furnished by the Chemico Laboratories, Indianapolis, Ind.

‡ We are indebted to Miss Helen Auth of this Laboratory for making the counts.

TABLE I. Mortality and Time to Death of Mice Treated with Reticulose or Reticulose and Streptomycin, and of Rats Treated with Reticulose, Compared to Controls.

Treatment*		No. of animals	% mortality (28 days)	Mean time to death ± S.E.
Mice				
625 r, R	Not bled	29	90	13.2 ± .6
	Bled	40	78	12.1 ± .6
	Total	69	83	12.6 ± .4
625 r, C	Not bled	30	77	12.5 ± .6
	Bled	40	90	12.1 ± .5
	Total	70	84	12.3 ± .4
625 r, S, R	Not bled	29	69	15.8 ± 1.3
	Bled	39	67	13.9 ± .9
	Total	68	68	14.7 ± .8
625 r, S, C	Not bled	30	50	14.2 ± 1.1
	Bled	40	72	14.3 ± .8
	Total	70	63	14.3 ± .6
Rats				
570 r, R	Not bled	36	75	11.8 ± .9
570 r, C	" "	36	75	11.6 ± .9

* R = Reticulose; C = Control; S = Streptomycin.

TABLE II. Leucocyte Counts from Tail Blood of Reticulose-Treated Mice, with or without Irradiation or Streptomycin Treatment, Compared with Controls.

Treatment group*		Leucocytes, thousand/mm^3 (Day after beginning Reticulose; † No. of mice)			
625 r	R‡	5.24 ± .37 (0; n = 20)	2.02 ± .15 (2; n = 20)	1.05 ± .07 (6; n = 19)	.77 ± .07 (13; n = 19)
	C	4.91 ± .34 (0; n = 20)	2.60 ± .09 (2; n = 19)	1.40 ± .06 (6; n = 20)	.92 ± .17 (13; n = 15)
	S, R	5.40 ± .27 (1; n = 20)	1.73 ± .08 (3; n = 19)	.61 ± .07 (7; n = 18)	.53 ± .07 (14; n = 23)
	S, C	4.84 ± .36 (1; n = 20)	1.44 ± .08 (3; n = 20)	.42 ± .05 (7; n = 19)	.56 ± .06 (14; n = 18)
No irrad., R		14.58 ± .56 (0; n = 29)		12.49 ± .47 (8; n = 27)	11.24 ± .38 (15; n = 27)

* Reticulose (.1 ml) and streptomycin (5 mg) subcutaneous injections begun the day after irradiation and continued for 16 days.

† Day 0 = 1 day after irradiation of the first four groups, and just prior to the first Reticulose injection.

‡ R = Reticulose; C = Control; S = Streptomycin.

± 1,200 for tail blood the day following injection) there were, unfortunately, no tail-blood counts made on the controls (mean for heart blood = 5,450 ± 850). Wide differences in leucocyte counts from heart and tail blood are generally acknowledged. For example, Fekete(5) reports the average value for tail blood of 8 normal mice to be 21,510 cells/cmm compared with 3,717 for ventricle blood. In the data of Thompson(2) on leucocyte and lymphocyte counts in rabbits the differences appear to be in excess of expected chance variation.

Although it has not been established that an increased number of circulating lymphocytes improves the chance of survival after irradiation (Campbell and Ross)(6), the possibility remained that a very small improvement in the animal's natural defenses might enhance the saving effect of antibiotic treatment. Reticulose treatment did not accomplish this in mice where streptomycin treatment was effective.

It seems somewhat futile to attempt an explanation of the discrepancy between our results and the conclusions drawn by Thompson(2), and Berry and Mitchell(1), but their procedures differed from ours in several respects. In their rabbits Reticulose was given intramuscularly and in their mice, intraperitoneally; the rabbits were given 2 ml each on days 2 and 3, and the mice were given a single injection of 0.15 ml; and the radiation dose given rabbits (450 r) was in the low lethal range. The response of irradiated rabbits to treatment with Reticulose, which was small though apparently significant, may be different from that of rats or mice.

Whether or not a real difference exists between the experimental results from the laboratories concerned, the fact that survival was not increased in irradiated rats and mice, and that no augmentation of the streptomycin effect occurred in mice, indicates to us that no spectacular results are to be expected from the use of Reticulose following whole body exposure to radiation.

Summary. Reticulose given by subcutaneous injection to mice, with and without streptomycin treatment, failed to alter mortality, leucocyte count, or incidence of blood stream infection after 625 r whole-body X-irradiation. Given to rats it failed to alter mortality after 570 r whole-body X-irradiation.

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Routine Titrations of Lansing Virus and Antibody in Mice: Comparison of Methods of Estimating Endpoints.* (19796)

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The reproducibility of endpoints in titrations of Lansing virus and antiserum has been discussed by Morgan(1) and Bell(2), who present the results of relatively short series of independent titrations from which the ED_{50}'s have been calculated. These estimates seem very consistent, considering the limited numbers of mice on which they are based. Similar results have been obtained by Schwerdt and Merrell(3) in cotton rats. In all 3 reports the ED_{50}'s have been estimated by the Reed and Muench method. The relative advantages of estimating the ED_{50} by this and other methods have been reviewed by various workers, notably Finney(4), who suggests that the Kärber method may be expected to give very satisfactory results when responses run from 0 to 100%. An advantage of the Kärber method under these conditions is that the standard error of the estimate may be readily calculated; in contrast, the procedure outlined by Pizzi(5) for determining the standard error of Reed and Muench estimates seems arbitrary. The purpose of this note is to present some results obtained in these laboratories in the routine titration of Lansing virus suspensions and antisera. The ED_{50}'s of the titrations have been calculated by a number of different methods in order to compare the estimates obtained.

Materials and methods. Over a period of several months, one of us (E.M.C.) carried out routine titrations of a pool of Lansing virus, and of samples of single lots of plasma gamma globulin, placental gamma globulin, and immune monkey serum. The virus pool

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TABLE I. Routine Titrations of Lansing Virus.

	No. of titrations	% mortality of mice at following dilutions (log units): −5.2	−4.7	−4.2	−3.7	−3.2	−2.7	Mice per dilution
Virus pool	20	18	25	38	65	75	92	10
		Routine titrations of Lansing Antisera*						
		−5	−4.5	−4	−3.5	−3	−2.5	
Plasma γ globulin	9			76	70	33	6	8
Placental γ globulin	8		88	78	38	4		8
Monkey serum	12	93	83	51	13	11		8

* Dilution of globulin or serum mixed with equal volumes of virus to give a final virus concentration of $10^{-2.7}$.

TABLE II. Reproducibility of ED_{50}'s (Log Units) Estimated by the Probit Method.

	No. of titrations	Avg ED_{50}	Range of ED_{50}'s	Stand. dev. of ED_{50}'s	Avg stand. error of individual ED_{50}
Virus pool	20	−4.06	−4.72 to −3.38	.35	.22
Plasma γ globulin	9	−3.36	−3.99 to −2.94	.36	.17
Placental globulin	8	−3.71	−3.87 to −3.47	.14	.14
Monkey serum	12	−3.98	−4.38 to −3.44	.32	.16

was prepared from the brain stems and spinal cords of approximately 300 mice inoculated intracerebrally with Lansing virus as previously described(6). The gamma globulins were commercial preparations formerly used by us in protection tests(7) and containing at least 16% gamma globulin; the monkey serum was obtained following a course of intramuscular injections of Lansing virus(8). The virus pool and monkey serum were distributed into screw-capped vials and stored in a carbon dioxide chest; the gamma globulins were stored in packages at 4°C. The gamma globulin and monkey serum were diluted in normal saline for the tests, and held at 4°C overnight. An equal volume of Lansing virus pool, diluted in normal saline, was then added to each serum dilution to give a final concentration of virus of $10^{-2.7}$. The dilutions of the materials used, the number of mice per dilution, and the number of titrations carried out are given in Table I. The mice were inoculated cerebrally with 0.03 ml of each suspension; they were all males, 12 to 16 g in weight obtained from the colony maintained at these Laboratories. The inoculated mice were observed daily for 4 weeks and all deaths recorded. Deaths occurring on the day after inoculation were not regarded as specific. The average percentage mortalities are given in Table I. It will be noted that these percentages did not usually run from 0 to 100%, but that considerable truncation often occurred at one or both extremes. Included among the titrations were several in which the responses were very erratic.

Statistical methods. Estimates of the ED_{50}'s and their standard errors were calculated for the individual titrations by the probit method(9). Two iterations were carried out in all cases. In addition, the ED_{50}'s were calculated by the following procedures: Kärber(10); Reed and Muench(11); and moving average(12). The maximum possible span was used in determining the ED_{50}'s by this last procedure.

Results. The ED_{50}'s obtained by the probit method are summarized in Table II. The variability of these quantities is considerable, the estimates differing among themselves as much as one log unit or more. The standard deviation of the titrations is generally greater than the error of the individual assay, suggesting that titrations carried out on different occasions vary more than those performed at the same time. The average slope is 0.93 probit per log unit for the virus assays and 1.6 probits per log unit for the serum titrations.

TABLE III. Comparison of ED_{50}'s Obtained by Various Simple Methods with Those Obtained by the Probit Method.*

	Kärber		Reed & Muench		Moving avg	
	Max diff	Avg diff	Max diff	Avg diff	Max diff	Avg diff
Virus pool	65	23	63	29	63	30
Plasma γ globulin	31	19	72	42	56	31
Placental globulin	47	12	110	36	53	23
Monkey serum	64	16	64	25	68	27

* Differences as percentages of stand. errors of probit method.

A comparison of the estimates obtained by the Kärber, Reed and Muench, and moving average methods with those obtained by the probit procedure is given in Table III. The differences in log units are expressed as percentages of the standard errors of the individual assays, as calculated by the probit method. None of these methods gives widely different results, on the average, from those of the probit method; several of the largest discrepancies occur for titrations that cannot be considered satisfactory. The Kärber method appears to give noticeably better estimates than the others, differing on the average by less than one quarter of a standard error.

A comparison was also made of the standard errors of the individual asays as estimated by the probit and by the Kärber methods. In general, it was found that the agreement was quite satisfactory, providing the truncation was not too great. For titrations with no more than 20% mortality at the one extreme or no less than 80% at the other, the Kärber estimates were found to differ by 12%, on the average, from the probit estimates. In general, they tended to be smaller than the probit estimates, appreciably so if the truncation was over 25%.

Discussion. As judged by these results, titers of virus or immune serum based upon 50-60 mice are subject to considerable variation. This variation is greater than that reported by Morgan(1) and Bell(2), and that expected from the results of our individual titrations. For this reason it would be unwise to use the standard error of the individual assay for comparing ED_{50}'s determined on different occasions, as advocated by Schwerdt and Merrell(3). While the accuracy of comparisons could be improved by increasing the number of animals, it should be noted that a 4-fold increase in number would be required to reduce the standard error of the individual titration by one half. This, of course, would not affect variation from one time to another. The most accurate comparisons would be made with titrations done on the same occasion. Since the average slope of the dosage response curves of the serum assays is considerably greater than that of the virus, serum titers can be determined a good deal more precisely than virus, *i.e.*, approximately one third the number of animals are required for the same accuracy.

The comparisons tabulated above suggest that the ED_{50}'s calculated in routine assays by the Kärber method compare very favourably with those obtained by the probit method. Providing the response runs from 0 to 100%, both ED_{50}'s and their standard errors as given by this simple procedure will agree very closely with those of the more involved maximum likelihood method. If, then, care is taken to avoid instances where extreme truncation occurs, particularly those in which the ED_{50} lies far from the center of the dilution series, the estimates of this quantity obtained by the Kärber method should be quite satisfactory, and likely more accurate than those obtained by the Reed and Muench procedure. In addition, the arithmetic of the Kärber method is somewhat simpler than that of the Reed and Muench procedure. It would seem then that the Kärber method is to be preferred in the estimation of ED_{50}'s from this type of data.

Summary. 1. Over a period of several months, routine titrations in mice were carried out by a single worker of a pool of Lansing virus, and of samples of plasma gamma globulin, placental gamma globulin, and immune monkey serum. Estimates of the ED_{50}'s and their standard errors were calculated for the individual titrations by the probit method. In addition ED_{50}'s were calculated by the

Kärber, Reed and Muench, and moving average methods. 2. The standard deviation of the titrations was found to be greater than the error of individual assays, suggesting that titrations carried out on different occasions vary more than those performed at the same time. 3. None of the methods mentioned gave widely different estimates of the ED_{50}. In particular, the ED_{50}'s calculated by the Kärber method compared very favourably with those obtained by the probit method. 4. The Kärber method is proposed as a suitable alternative to the Reed and Muench procedure for the calculation of ED_{50} in routine titrations of virus and antiserum.

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Effect of X-Ray on Radioactive Phosphorus Turnover and Oxygen Consumption of Brain.*† (19797)

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Studies of the pathological effects of radiation on the central nervous system of adult mammals have shown no clearcut findings. Warren(1) presents evidence which indicates that at least "therapeutic radiation" has little effect on adult nerve tissue. Findings which demonstrate radiation damage to nervous tissue appear to result from effects on vascular components rather than direct damage to neural elements. The earliest experimental observations(2) of the effects of radiation on brain were in experiments where the whole body was irradiated; congestion and hemorrhage were the most consistent pathological findings.

The purpose of the present investigation was to demonstrate the presence or absence of alterations in the phosphorus metabolism of brain after whole-body irradiation with roentgen rays. In order to eliminate at least one variable, vascular circulation effects, studies of oxygen consumption and phosphorylation were done *in vitro* after *in vivo* irradiation of the animals.

Experimental. Six- to eight-week-old male albino mice were irradiated with 500 to 800 r and, in another experiment, with 19,000 r in air. A 250 kv General Electric Unit was used with 1 mm A1 and 0.5 mm Cu filters at 15 ma output. The smaller dosages were delivered at 1 meter distance and the greater at 7 cm. The mice were killed by severing the spinal cord at various times after irradiation. The brain was excised as rapidly as possible and split along the longitudinal fissure into equal halves. The halves were weighed, minced, and then added to 3.0 cc of McIlwain's buffer(3) containing P^{32} as orthophosphate.‡ The tissues were incubated at 37.5°C for 3 hours

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TABLE I. Qo_2 and Phosphorus Uptake of Brain as Affected by X-irradiation.

Exp.	Dosage (r)	Hr post irrad.	No. samples	Qo_2 (µl/h) †	Uptake as % of incubation medium: Acid sol.	Residual	Lipid
1	500	19	7	452 ± 30*	.80 ± .08	.060 ± .008	.036 ± .008
	0	19	7	448 ± 46*	1.16 ± .16	.074 ± .008	.042 ± .008
2	500	0	6	378 ± 30	1.26 ± .22	.088 ± .006	.054 ± .006
	500	190	6	396 ± 30	1.76 ± .12	.104 ± .014	.062 ± .010
	0	0	6	386 ± 36	1.74 ± .18	.098 ± .002	.056 ± .008
3	800	18	6		1.1 ± .2	.15 ± .01	.12 ± .02
	0	18	6		1.2 ± .1	.12 ± .02	.09 ± .02
4	800	0	6		1.3 ± .2	.14 ± .02	.11 ± .01
	0	0	6		.95 ± .08	.12 ± .03	.12 ± .02
5	800	0	4		1.2 ± .3	.10 ± .005	.12 ± .01
	0	0	4		1 ± .05	.10 ± .008	.12 ± .01
6	19000	18	8		1.5 ± .2	.13 ± .01	.060 ± .008
	0	18	4		1.3 ± .3	.12 ± .01	.055 ± .007

* Extrapolated from 15 min. value.

† Results expressed as per 200 mg fresh brain tissue.

There was no significant difference in phosphorus turnover and oxygen consumption between the right and left hemispheres of the same animal. P from Fisher's(6) table of t was $<.1$ for protein and $<.8$ for the lipid fractions where $n = 19$. For oxygen consumption $p < .4$ where $n = 9$.

in either a Warburg apparatus under an atmosphere of oxygen (Exp. 1-2), or 3½ hours on a Dubnoff metabolic shaker under Carbogen (Exp. 3-6). An equal number of unirradiated brain halves were used for controls in each experiment.

Metabolic processes were stopped at the end of the incubation period by the addition to the media of an equal volume of 10% trichloracetic acid. The resulting suspension was fractionated by the procedure described by Rafelson(4) into acid-soluble, lipid and residual "protein" fractions.

Results and discussion. Data presented in Table I indicate that, at the radiation levels employed, the capacity of adult mouse brain tissue to utilize oxygen and to metabolize exogenous phosphate is not significantly impaired.

Quastler *et al.*(5) have reported that whole body irradiation at levels ranging from the minimal lethal dose to 10,000 r resulted in death within 3.5 days from effects on the gastrointestinal system, mainly hemorrhage. Doses of X-ray exceeding 16,000 r, however, produced death within an even shorter period after exposure from what appeared to be central nervous system involvement. Irradiation of the head alone was sufficient to produce the "hyperacute" radiation death. Yet, the present study, in which doses of radiation as high as 19,000 r were used, demonstrated no alteration of phosphorus metabolism.

It is possible that the lethal effects of such doses are associated with phases of cellular metabolism unrelated to phosphorylation mechanisms. However, if phosphorus turnover were a valid index of the metabolic state of a tissue, it would appear that there is no acute gross derangement of brain metabolism even after such large amounts of irradiation.

Conclusion. In vivo irradiation with 500 to 800 r of X-rays did not impair the ability of adult mouse brain tissue to metabolize exogenous radiophosphate and did not alter the Qo_2 of the minced tissue. Doses of 19,000 r produced no observable acute effect on the phosphorylation.

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Ultraviolet Radiation and Streptomycin-Resistant Mutants. (19798)

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The origin and nature of antibiotic-resistant bacterial variants has been the subject of considerable discussion. A recent report has cast doubt upon a considerable amount of experimental data which has been presented in many reports concerning the enhancement of resistance to antibiotics following ultraviolet irradiation(1). These workers reported that they obtained no enhancement in resistance to streptomycin or penicillin following ultraviolet irradiation of *Micrococcus aureus*, although such was observed following irradiation of *Escherichia coli*. It was highly desirable to reinvestigate this matter, and the present report covers some of the data obtained.

Experimental. Micrococcus aureus PCI was grown in nutrient broth at pH 7.2 and 18 ml of 12-hour cultures were beaten for 30 seconds in a Waring Blendor as described below, and then irradiated for 240 seconds in sterile Petri dishes which were gently agitated to insure uniform irradiation. A GE germicidal lamp, emitting about 90% 2537 Å, was used at a distance of 3 inches from the irradiated culture. Appropriate dilutions were plated upon nutrient agar (Difco) containing varying concentrations of streptomycin sulfate for determination of spontaneous and zero point mutation incidences. Flasks of nutrient broth (Difco) were inoculated with 5 ml of the irradiated culture for determination of mutation incidences after periods of growth. Streptomycin was added to the sterile media (45°C) and the agar was then thoroughly stirred. Irradiation, dilution and plating procedures were carried out rapidly in a darkened room to minimize the various recovery phenomena, *e.g.*, photo- and heat-reactivation, recovery upon standing in dilution fluid, etc. Nonirradiated aliquots of each culture were treated similarly for controls. Plating was carried out in triplicate except at levels of streptomycin above 6/ml, where 6 plates each were used. All plates were incubated 6 days for final counts.

The pronounced clumping tendencies of *M. aureus* present a problem of considerable magnitude in interpreting the results of viable count determinations. A single resistant cell in a clump of 20 will yield exactly the same results upon plating as would have been the case had all 20 been resistant. To determine the relation of resistant cells to clumps, aliquots of each group were placed in a sterile Waring Blendor and beaten for 30 seconds with the motor cut to half speed by means of a variable rheostatic control. Average cells/clump were also determined microscopically in each case by counting all cells and clumps in 30 fields.

Results. Fig. 1 shows the streptomycin-resistance distribution of *M. aureus* before and after irradiation. Only spontaneous and end-point mutants are shown (24 hours incubation) since the radiation dose used (240 seconds) killed 99.998% of the cells. This reduced the number of viable organisms to such an extent that it was possible to determine the zero-point mutation incidence for only the very low levels of streptomycin(2). The percentages indicated in the ordinate are the percent survivors referred to the total number of viable cells at the time of test. Treatment in the Waring Blendor increased the colony count 3-fold although average clump size as determined by microscopic count was reduced from 6.4 to 1.4 cells/clump. The colony counts upon streptomycin agar were not increased by the treatment.

Discussion. The data presented support the several reports that there is an increase in incidence of resistant organisms following a heavy dose of ultraviolet radiation. The failure of Saz, *et al.*(1), to observe such an increase was undoubtedly due in large part to the low dosage used, since only 60% of their *M. aureus* cells were killed(3). With *E. coli*,

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* Post-doctoral Fellow in Cancer Research of the Damon Runyon Memorial Fund.

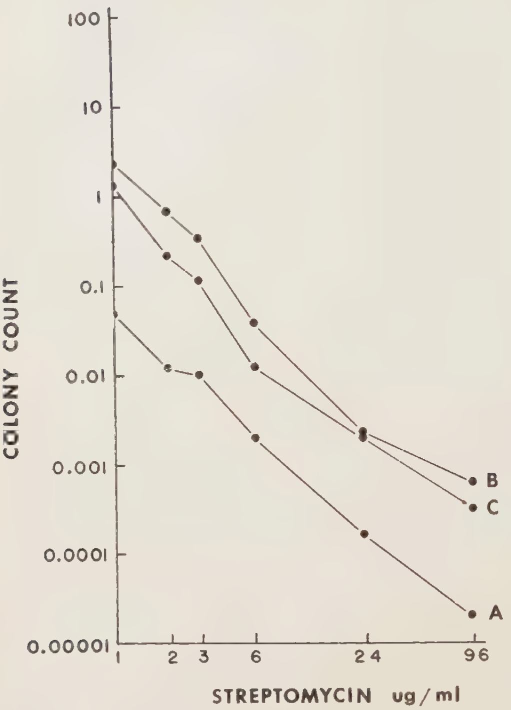

FIG. 1. Comparison of resistance to streptomycin of irradiated and nonirradiated *Micrococcus aureus* PCI. Colony count is referred to control without antibiotic as 100. Curve A: Preirradiated culture with clumps broken so as to yield clumps containing 1.3 cells; total organisms, 2.74 billion/ml. Curve B: End point mutants, 24 hr incubation following irradiation which killed 99.998% of the culture reported in Curve A; inoculum, 5 ml containing 47,000 viable cells/ml into 250 ml; total viable organisms (clump size 6.4 cells), .508 billion/ml. Curve C: Same culture as that reported in Curve B except clumps broken; total organisms after beating, 1.41 billion/ml.

where a dose sufficient to kill 99% of the culture was used, they obtained an increased incidence of resistant organisms.

The problem of clumping is crucial in making determinations of this nature. A large clump may consist entirely of resistant cells, or only one in the clump may be resistant. In either case, it will appear on the streptomycin agar plate as a resistant clone, and may thus drastically alter results obtained. In the experiments reported here, average clump size was 6.4 cells/clump. Following brief beating, it was reduced to 1.4. The treatment apparently reduced viability of the suspension, since the plate counts showed an increase of only 3-fold. The influence of this treatment, however, upon the actual number of resistant organisms was negligible. This would seem to indicate that resistant cells were either not contained in large clumps, or that the clumps formed were very tightly bound, and therefore, resistant to being broken by the beating. Irradiation of an organism which may form some clumps containing from 50 to 100 cells, cannot be uniform since those in the interior would receive very little radiation, even following prolonged treatment.

It is of interest that following irradiation there was a proportional increase in numbers of resistant organisms at all levels tested. Many factors besides clumping have been shown to markedly influence the actual number, and incidence, of antibiotic resistant mutants(4). The influence of several of these is indirect, such as the pH employed with antibiotics which show different activity as the pH changes, or where a considerable part of the antibiotic is absorbed non-specifically by the colloidal particles of the medium.

Summary. Data have been presented confirming earlier reports of an increased incidence of streptomycin-resistant organisms following a heavy dose of ultraviolet radiation. The importance of clumping characteristics in such determinations is emphasized by the fact that treatment designed to break the clumps results in an increased viable count, but not in an increase in actual number of resistant organisms.

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Chemical and Enzymatic Changes in Liver Following Freezing-Drying and Acetone Fixation.* (19799)

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The superior quality of frozen-dried preparations for histochemical and cytochemical localization of substances compared to chemically fixed tissues has been reported by many investigators. Theoretically, tissues prepared in this manner should show minimum changes from the original fresh tissues both chemically and structurally, except those physico-chemical alterations induced by freezing-drying itself. Whether such ideal conditions persist after paraffinization, deparaffinization, and hydration of frozen-dried tissues still needs to be investigated. Attempts at chemical analyses of frozen-dried material have been relatively few and of limited scope(1-4).

In the present investigation chemical and enzymatic assays were made on tissues in different stages of preparation in the freezing-drying procedure(5). Values from fresh control tissues were compared with those after freezing, freezing-drying, and freezing-drying followed by paraffin embedding, deparaffinization, and hydration. Further studies were made on the individual effects of heat and xylol on frozen-dried tissues. Comparative studies were also made on tissues prepared by cold acetone fixation, which is frequently used in histochemical studies. Information on quantitative effects of acetone fixation has been limited(2,6).

Materials and methods. Livers were obtained from normal strain A mice which were 12 ± 2 weeks old. Samples of tissue were taken from pooled, sliced left lateral and median lobes of 3 or 4 animals. In each experiment fresh samples weighing .4-1 g were taken. One sample was always analyzed immediately for control values, the others were sliced further into thin pieces (.5-1 mm in thickness), subjected to procedures used for histological preparations and then analyzed chemically. For freezing-drying, pieces were frozen individually in isopentane cooled by liquid nitrogen. They were dehydrated *in vacuo* at —25° to —30°C(7) over a period of 3 to 4 days. To observe the effects of freezing alone, tissues frozen as above were wrapped in aluminum foil and stored in a sealed test tube at —30°C for 3 days. The storage mixture consisted of 95% ethyl alcohol and dry ice. In experiments with paraffin embedding, frozen-dried tissues were infiltrated with paraffin at 60°C for ½ hour. To approximate the effects of heat employed in this procedure, separate samples of frozen-dried tissues were placed in a desiccator in the oven under comparable conditions. For acetone fixation pieces comparable in size to those for freezing-drying were placed in absolute acetone at 6°C for 3 days. Three changes were made on the first day and one on each of the following days. Some samples were analyzed directly after this procedure. Others were treated with 2 changes of chloroform for 1 hour at room temperature and infiltrated with paraffin as described. Paraffin blocks of frozen-dried and acetone-fixed tissues were sectioned at 25 μ thickness soon after embedding. Sections were stored overnight in sealed centrifuge tubes in the refrigerator at 4-5°C and were analyzed after deparaffinization and hydration by washing and centrifuging in the following solvents at room temperature: xylol, 30 minutes (5 changes); acetone, 30 minutes (4 changes); 70% acetone, 15 minutes (2 changes); distilled water, 15 minutes (3 changes). This schedule, necessarily longer than the usual histological procedure because of the thicker sections and larger quantities of tissue involved, was strictly followed. Additional experiments were carried out to determine the effects of xylol used in extracting the paraffin. Frozen-dried samples were homogenized with 3 successive portions of xylol, which

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TABLE I. Percentage Recovery of Chemical Constituents of Liver Following Various Treatments.

	No. of	Total		Acid soluble		Lipid soluble		Protein		
Treatment	samples	N	P	N	P	N	P	N	PNA	DNA
Freezing	5	95±1§	93±2	100±1	96±4	96±8	90±2	94±2	96±2	95±4
FD*	6–14	89±2	90±2	101±3	86±2	82±7	89±1	90±2	87±3	87±3
FD-xylol	6	89±2	86±4	105±7	98±7	86±5	82±5	86±3	88±4	90±2
FD-heat	10–14	92±2	90±2	97±4	94±4	98±5	87±3	89±2	86±1	93±3
FD-P†	6–10	69±3	29±2	25±4	14±3	36±9	28±3	83±3	26±2	80±2
Acetone	5–8	75±2	60±2	33±4	25±2	85±4	56±4	84±2	81±3	94±6
Acetone-P‡	6–10	67±3	26±2	14±2	6±1	38±6	6±2	79±3	78±4	71±3

* Freezing-drying. † Freezing-drying followed by paraffin embedding, deparaffinization, and hydration. ‡ Acetone fixation followed by paraffin embedding, deparaffinization, and hydration.
§ Stand. error. N = nitrogen; P = phosphorus.

TABLE II. Percentage of Chemical Constituents of Liver Based on Protein Nitrogen and DNA.

	Total		Acid soluble		Lipid soluble		Protein		
Treatment	N	P	N	P	N	P	N	PNA	DNA
Freezing									
Protein N	101	99	106	102	102	96	100	102	101
DNA	100	98	105	101	101	95	99	101	100
FD									
Protein N	99	100	112	96	91	99	100	97	97
DNA	100	101	113	97	92	100	101	98	100
FD-xylol									
Protein N	103	92	122	114	100	95	100	102	104
DNA	99	95	116	109	95	91	95	97	100
FD-heat									
Protein N	103	101	109	105	110	97	100	96	104
DNA	99	97	104	101	105	94	96	92	100
FD-P									
Protein N	83	35	30	17	43	34	100	31	96
DNA	86	36	31	18	45	35	104	32	100
Acetone									
Protein N	89	71	39	30	101	67	100	96	112
DNA	80	64	35	26	90	59	89	86	100
Acetone-P									
Protein N	85	33	18	8	48	8	100	99	90
DNA	94	37	20	8	54	8	111	110	100

See Table I for explanation of abbreviations.

was finally removed by evaporation under reduced pressure at room temperature. The various samples prepared by the foregoing procedures were homogenized with cold 0.85% saline and made up to volume in a 10 ml volumetric flask. Aliquots were assayed for acid and alkaline phosphatase(8), esterase (9), succinoxidase and cytochrome c oxidase (10). In addition aliquots were fractionated according to the method of Schneider(11) and each fraction was analyzed for nitrogen (micro-Kjeldahl) or phosphorus(12) or both. Analyses were also performed for desoxypentose nucleic acid, DNA(13), and pentose nucleic acid, PNA(14).

Results. The effects of histological procedures upon the various chemical constituents of mouse liver are presented in Tables I and II. Table I shows the results in percentages of the original content, as determined from the fresh weight of the tissue and the values obtained in the control sample. In Table II an attempt has been made to correct for losses associated with manipulation of the tissue before it is ready for chemical analysis. Such losses occur because of adhesion of tissue to the razor blade in cutting a large number of small pieces, adhesion of tissue to forceps, and fragmentation of tissue during the freezing operation. This correction was made by calculating the results on the basis of recovered DNA and protein nitrogen, assuming that these 2 components would be least affected by the subsequent washing procedures employed.

TABLE III. Effect of Various Histological Procedures on Enzymes of Mouse Liver, Expressed as % of Control Based on Protein Nitrogen.

Treatment	No. of samples	Alkaline phosphatase	Acid phosphatase	Succinoxidase	Cytochrome oxidase	Esterase
Freezing	5	110 ± 8*	87 ± 6	52 ± 9	33 ± 4	88 ± 6
FD	14	87 ± 5	92 ± 4	37 ± 4	56 ± 8	91 ± 5
FD-xylol	6	111 ± 6	108 ± 4	70 ±20	39 ± 8	96 ± 9
FD-heat	14	94 ± 5	92 ± 5	29 ± 7	58 ±11	76 ± 5
FD-P	10	29 ± 5	13 ± 6	0	0	10 ± 2
Acetone	8	49 ± 3	49 ± 6	0	0	26 ± 2
Acetone-P	10	26 ± 2	11 ± 1	0	0	11 ± 3

* Stand. error. See Table I for explanation of abbreviations.

This assumption seems to be justified since the recoveries were essentially the same when referred to these different baselines.

Freezing, freezing-drying, and freezing-drying followed by heating or xylol treatment had little or no effect on most of the nitrogen and phosphorus containing fractions of mouse liver (Table II). There was, however, a relative increase in the amount of acid soluble nitrogen, which may be due to degradation of protein during the freezing or freezing-drying process, resulting in an increase in the acid soluble material relative to the protein. The elevated content of acid soluble nitrogen relative to DNA may be explained by assuming that either DNA was incompletely extracted or that partial degradation of nucleic acid has occurred, resulting in a relatively lower DNA content in the nucleic acid fraction. Acetone fixation resulted in considerable removal of acid soluble and lipid soluble material. The effects of paraffin embedding, deparaffinization and hydration were equally severe on acetone-fixed and frozen-dried tissue, and resulted in substantial loss of acid soluble and lipid soluble nitrogen and phosphorus. There was also a significant loss of PNA in frozen-dried tissue which had been subjected to the deparaffinization-hydration process.

Freezing of tissues, freezing-drying, and freezing-drying followed by xylol or heat produced little or no effect on the acid and alkaline phosphatase or esterase but resulted in substantial loss of succinoxidase and cytochrome c oxidase activity (Table III). Freezing-drying followed by paraffin embedding and deparaffinization-hydration resulted in complete loss of succinoxidase and cytochrome c oxidase and considerable reduction in phosphatase and esterase activity. Acetone fixation completely destroyed the oxidase activity and caused a marked decrease in the activity of the other enzymes.

Discussion. Liver tissue prepared by the 2 principal methods now employed for microscopic demonstration of enzymes has been analyzed quantitatively for various chemical constituents and enzyme activity at different stages of preparation. The results obtained confirm previous claims that little change occurs in the chemical composition of frozen-dried tissues. Frozen-dried preparations are chemically similar to frozen controls. Of the enzymes studied the phosphatases and esterase appear to be unaffected both by freezing and freezing-drying. Doyle(2) has reported high activity for phosphatases in frozen-dried lymph nodules. The process of freezing and storage, however, is sufficient to cause a reduction in oxidase activity. This is in agreement with the results of Novikoff and Potter (15), and Nowinski and DeRobertis(3).

It is evident that in frozen-dried tissues much of the enzyme activity and soluble constituents are lost during the paraffin embedding, deparaffinization, and hydration procedure. Since neither the heat nor xylol treatment affects enzyme activity appreciably, it must be assumed that the loss of activity is associated with either the hot paraffin treatment or the repeated washing procedure subsequent to the xylol treatment, or both. Although the effects of graded alcohol series for hydration were not investigated, losses similar to those which occur during graded acetone hydration may be expected since the 2 procedures are essentially alike. The time for hydration of tissue subsequent to paraffin embedding was longer in the present investigation than is normally used in routine histochemistry; this was necessary because of the larger amount of tissue used. The conditions may be comparable, however, since in the usual histochemical technics diffusion may be more rapid than in the larger pieces of tissue used here. It follows, then, that better results would be obtained in microscopic sections if this hydration sequence could be avoided. It has been observed(16) and confirmed in this laboratory that in the histochemical demonstration of acid phosphatase(17) considerably stronger reactions are obtained when non-deparaffinized sections are employed. Shorter incubation times are required and localization seems to be better. It has also been observed (18,19) that even with deparaffinized tissues, the microscopic appearance of frozen-dried tissues is superior to that of acetone-fixed tissues, although quantitatively both materials are equivalent in enzymatic activity (Table III).

The data presented here show that the value of freezing-drying for histochemistry may be diminished by the subsequent treatment of the tissues. It is desirable therefore to improve the methods of handling frozen-dried material. Emphasis should be placed on technics which avoid excessive contact of the tissues with solvents. Staining and histochemical enzyme procedures which require relatively short periods of immersion in aqueous solutions should also be preferable for retaining various substances. The use of carbowax(7,20) has been suggested.

Summary. 1. The chemical composition and enzymatic activity of mouse liver following procedures used in the preparation of tissues for histochemical studies show that high percentages of enzyme activity and chemical constituents are retained after freezing alone and freezing-drying. Negligible additional losses occurred in frozen-dried tissues as a result of a short heating period and treatment with xylol. Considerable loss of material and substantial reduction in the activity of acid and alkaline phosphatase and esterase occur after paraffin embedding, deparaffinization and hydration. Under the same conditions the activity of succinoxidase and cytochrome c oxidase is completely destroyed. 2. In acetone fixation, in contrast to freezing-drying, there is a greater initial loss of enzyme activity and soluble substances, with further loss upon deparaffinization and hydration.

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A Method for Detection of Leukocyte Injury Based on Release of a Lysozyme-like Enzyme.* (19800)

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Damage to cells is accompanied by the release of certain cellular contents into the surrounding medium. Various investigators, such as Rossiter(1), have invoked this process as a means of releasing enzymes from rabbit leukocytes for assay and study. Saponin, in particular, has been used to accomplish the liberation of enzymes from cells(1). The effect of hypotonicity was employed by Walker(2) in his studies on the β-glucuronidase content of mouse liver. In connection with studies in progress in this laboratory on the metabolism of human leukocytes, a need was found for a screening test for substances having an adverse effect on cellular metabolism. It seemed that the phenomenon of release of cellular contents into the surrounding medium occurring with injury of the cell might be utilized for this purpose. An intracellular enzyme with the properties of lysozyme was selected as the material to be measured. The method is described here, together with the effects observed on exposure of human leukocytes to varying concentrations of saline and to leukotoxin.†

Procedure and results. Leukocyte suspensions. Whole blood was obtained from human subjects by venipuncture and added to heparin (Liquaemin-Roche) in final heparin concentration of 4 mg %. Sterile siliconed (GE 9987) syringes and glassware were used throughout the preparatory procedures. Sedimentation of red blood cells was accomplished by a dextran method(3) modified in this laboratory by Dr. S. P. Martin and to be reported by him. The supernatant leukocyte-platelet suspension in plasma was centrifuged at 65 g for 7 minutes, a procedure which leaves the platelets and some leukocytes in suspension. The supernatant was taken off, and the sedimented leukocytes were then resuspended in the desired test medium. The final count (or in hypotonic solutions, the equivalent count) of leukocytes is noted in the tabulated data, in each instance. In the sample experiments summarized in the present report, the test media consisted of decreasing concentrations of saline (Table I), and leukotoxin 1:50 in saline (Table II). *Leukocyte lysate.* Sedimented leukocytes as prepared above were resuspended in distilled water. *Lysozyme substrate.* Bacto lysozyme substrate‡ stock suspension was prepared by subjecting a 4% suspension in citrate-phosphate pH 6.2 buffer (as used by Pringle(4)) to 8000 cycles/sec vibration in a Raytheon Oscillator 9 kc for 10 min. For final use, the stock was diluted 5.5 ml per 100 ml of buffer, to 30% light transmission.

Method. In each instance, 1.0 ml of the leukocyte suspension was added to 1.5 ml of lysozyme substrate, and an immediate reading was obtained at 540 mμ in a Coleman Junior Spectrophotometer (75 x 12 mm cuvettes), 100% transmission being set by use of a blank tube containing 1.0 ml of the cell suspension and 1.5 ml of buffer. All tests were run in duplicate. After exactly 20 minutes in a 37°C water-bath without agitation, a second reading was obtained.§ Percent lysis

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† An antiserum to human leukocytes prepared by and supplied through the courtesy of Dr. James Tullis of the Department of Physical Chemistry, Harvard Univ. Med. School.

‡ A dried preparation of *M. lysodeikticus,* supplied through the courtesy of Mr. H. W. Schoenlein of Difco Laboratories.

§ Initially all determinations were done at intervals of 5 to 10 minutes over a period of 1 hour. Subsequently, it was found that a single reading at 20 min. sufficed for the present purpose, although the method of recording repeated observations has yielded interesting information as regards the enzyme *per se.* Studies concerning the absolute lysozyme activity of the cells in various conditions and under various circumstances form a separate investigation which is in progress.

TABLE I. Effect of Decreasing Concentrations of Saline on Human Leukocytes, Leukocyte Lysate and Crystalline Egg-White Lysozyme.

Test material (enzyme source)		Suspending or diluting medium, % conc. NaCl .850	.668	.486	.304	.121	.061
Human leukocytes	(A), 6500/mm³	9.5*	15.8	46.3	54.1	77.6	
	(B), 6700/mm³	20.1	32	37	60.9	81.9	
	(C), 800/mm³	7.2	7.5	11.5	18.4	49.4	
Leukocyte lysate (C), 800/mm³(⌒)		41.7	48	52.4	54.6	48.1	54.3
Crystalline egg-white lysozyme, 3.6 μg/ml		54.6	56.7	59.6	56.1	61.9	55.8

* Recorded as % lysis of lysozyme substrate.

TABLE II. Effect of an Antiserum to Human Leukocytes on Human Leukocytes, as Indicated by Enzyme Release from the Cell.

Leukocytes from subject	% lysis of lysozyme substrate with added: Saline	Leukotoxin	Index of injury*
(A) 5000/mm³	21	50.4	138
(B) 6300	16.6	49.6	194
(C) 4300	10.7	25.7	136

* See text (Method) for derivation of index. An index greater than 24 indicates significant injury.

of the substrate was calculated by the formula: 100 minus (the optical density at 20 minutes, divided by the optical density at 0, times 100). When it is desired to obtain data on the *release of enzyme* from experimentally injured leukocytes as compared to control leukocytes, it is necessary to correct this value for the rate of the enzymatic reaction in the control tubes, since differences noted in a fast-moving system cannot be compared directly with those observed in a slow-moving system. The following index is computed in such circumstances to correct for speed of the reaction: (% lysis in the presence of leukocytes exposed to test substance) minus (% lysis in the presence of control leukocytes), divided by (% lysis in the presence of control leukocytes), times 100. Thus, the larger the index, the greater the lysis of substrate in the presence of test leukocytes, as compared to control leukocytes. Control experiments on the method, using 2 S.D. in the calculations, indicate that an index greater than ±24 is very significantly altered. If a cell-free source of enzyme is used (as in plasma titrations), the S.D. is less, and an index of ±12 is significantly altered. The use of this index of injury is illustrated in Table II.

The rate of the enzymatic reaction in control leukocyte suspensions has been found to be determined to a considerable degree by the injurious effect of the lysozyme substrate itself on the leukocytes during the 20 minute period of observation. It is affected, also, by the care with which the leukocytes are handled prior to the titration and by changes in electrolytes, pH, temperature and time. There is also variability in the reaction rate from day to day in the same individual and from individual to individual, although leukocyte suspensions of closely-approximated count and lysozyme substrate concentrations of identical amount are used in each determination. In all comparative situations, the aforementioned factors operate equally in test and control tubes, so that differences observed have been attributed to the addition of the test substance. When a substance is to be tested for its injurious effect on leukocytes, it has been found desirable to use a suspension of at least 5000 leukocytes per cmm to detect differences consistently. In most of the studies to be reported in later communications, suspensions of 5500-6500 leukocytes per cmm have been used. The present report includes lower counts, as in Table I to compare leukocyte lysate activity with that of a leukocyte suspension of equivalent count.

The method as an indicator of cell damage. The method outlined above has been used as a test for cell injury following the exposure of leukocytes to a variety of physical, chemical and biologic agents. Most of these observations will be the subject of later reports.

Table I is included here as an indication of the changes that are observed following exposure of normal human leukocytes (subjects A and B), leukocytes and leukocyte lysate (subject C) and crystalline egg-white lysozyme‖ to decreasing concentrations of saline. It will be noted that while no consistent change occurs with lysate or crystalline lysozyme, there is a steady increase in lysis of substrate in the presence of leukocytes as the leukocytes are exposed to decreasing concentrations of saline.

Table II compares the percent lysis of substrate in the presence of normal human leukocytes as compared to that observed in the presence of leukocytes from the same suspension after exposure to leukotoxin for one hour. The marked increase in lysis of substrate after exposure of the leukocytes to leukotoxin is evident.

Summary. A method is described for the detection of cell injury by the measurement of intracellular substances released upon injury. The cells studied have been human leukocytes, and the intracellular substance selected for measurement has been an enzyme with the properties of lysozyme. Application of the method is illustrated by data concerning the adverse effect of hypotonicity and of an antiserum to human leukocytes on the cells. In further studies beyond the scope of the present report, the method has been found to be useful in screening a variety of physical, chemical and biologic agents for adverse effect on the metabolism of human leukocytes, as well as for detecting reversal of such injurious effects by protective agents.

‖ Obtained through the courtesy of Armour and Co.

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Uracil in Growth and Pyrimidine Nucleotide Synthesis of *Lactobacillus bulgaricus* 09. (19801)

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Orotic acid or a potential source of the compound is required for rapid growth of *Lactobacillus bulgaricus* 09(1-4). Ureidosuccinic acid has small but definite growth-promoting activity(2). Ureides of a number of other mono- and di-carboxylic acids which could not be visualized to yield orotic acid were synthesized and found to be essentially inactive (5). In experiments with *Lactobacillus bulgaricus* orotic acid or ureidosuccinic acid labelled with C^{14} in the ureido carbon gives rise to C^{14}-labelled uridylic and cytidylic acids of the bacterial cell(6). A considerable amount of non-radioactive dilution was observed in the nucleotides isolated despite the essential nature of a utilizable pyrimidine source for growth. Somewhat analogous findings were reported simultaneously by Abrams (7) who observed that a purine-requiring mutant of *Saccharomyces cerevisiae* synthesizes adenine and guanine from 1-C^{14}-labelled glycine as readily as does the wild type parent strain with no purine requirement. It has been reported(1,2) that uracil, uridine, uridylic acid, cytosine, cytidine or cytidylic acid are inactive in satisfying the requirement of *Lactobacillus bulgaricus* 09 for a pyrimidine. These findings led to the hypothesis that nucleic acid synthesis in *Lactobacillus bulgaricus* 09, and possibly in other species as well, normally does not involve the pyrimidine nucleosides and nucleotides as intermediates but instead involves the participation of an orotic acid nucleoside and an orotic acid nucleotide. According to this concept decarboxylation of orotic acid would not occur prior to incorporation into a "modified" nucleic acid. More extended studies reported here have now shown that while uracil is inactive in satisfying the pyrimidine requirement of *Lactobacillus bulgaricus* 09 under the usual conditions of assay (1-3 days of incubation), by extending the period of incubation (3-7 days) uracil promotes nearly maximal growth. Response curves to orotic acid determined with uracil as a constituent of the basal medium are "catenary" in type. Similar response curves have been reported previously showing the behavior of a tryptophane-synthesizing strain of *Lactobacillus arabinosus* to tryptophane(8) or of a histidine-synthesizing mutant of *Escherichia coli* histidine-less to histidine(9-12). Ryan and Schneider(9-12) have shown that these "catenary" type curves are to be expected if it is assumed that: (a) the bacterial population under study consists of a mixture of metabolite-requiring and metabolite-synthesizing strains, (b) growth in the "blanks" is due to unrestrained growth of the synthesizing strain, and (c) conformity to the expected response curve at intermediate and high levels of the metabolite is due to the existence of a certain "selection pressure" favoring the metabolite-dependent strains.

In addition to demonstrating the type of curves obtained with *Lactobacillus bulgaricus* 09 in response to orotic acid determined with and without uracil as a component of the basal medium, data are presented in this paper on the utilization of C^{14}-labelled uracil for the synthesis of bacterial uridylic and cytidylic acids when *Lactobacillus bulgaricus* 09 is grown under the following conditions: (a) in the presence of C^{14}-uracil as the only known source of pyrimidine (requires prolonged incubation) and, (b) in the presence of equal amounts of unlabelled orotic acid and C^{14}-uracil (rapid growth of the organism).

TABLE I. Basal Medium.*

Acid-hydrolyzed, norit-treated, vit.-free casein	10	g
Tryptic-digested, norit-treated, vit.-free casein	10	
DL-tryptophane	400	mg
L-cystine	200	
Glucose	40	g
Sodium acetate · 3 H_2O	20	
Adenine	10	mg
Guanine	10	
Xanthine	10	
Salts A	10	ml
Salts B	10	
Thiamine	2	mg
Riboflavin	2	
Nicotinic acid	2	
Ca pantothenate	2	
Pyridoxine	4	
PABA	1	
Biotin	10	γ
Folic acid	500	
Pyridoxal	500	
Vit. B_{12}	.4	
Tween 80	2	ml
pH to 5.8		

* Quantities given are for the preparation of one liter of double strength medium.

Experimental. *Lactobacillus bulgaricus* 09 was maintained as described in previous papers(1,2). Other procedures were according to customary technics with lactic acid bacteria (13). The basal medium had the composition given in Table I. Where uracil was used as a component of the basal medium it was present to the extent of 10 mg per liter of double strength medium. Titration of acid production using bromthymol blue as an indicator was used as a measure of the extent of bacterial growth. In the radioisotope experiments, *Lactobacillus bulgaricus* 09 was grown in 500 ml amounts of medium supplemented with 5 mg of C^{14}-uracil where this compound was used alone to promote growth and with 5 mg of orotic acid and 5 mg of C^{14}-uracil where the 2 compounds were used together. Thus these experiments were comparable in their design to those reported earlier in a preliminary note(6) where *Lactobacillus bulgaricus* 09 was grown with 5 mg of C^{14}-labelled orotic acid or with 15 mg of C^{14}-labelled DL-ureidosuccinic acid. The 2-C^{14}-labelled uracil was synthesized by the condensation of C^{14}-urea with malic acid according to a modification of the method of Johnson and Flint(14). Following the growth of *Lactobacillus bulgaricus* 09 at 37°C for 3 days with the combined supplement of orotic acid and C^{14}-uracil and for 7 days with the C^{14}-uracil alone the cells were centrifuged and washed once with water. The washed cells then were dried in a desiccator over Drierite. The dry cells (about 150 mg) were finely ground in the presence of 0.2 ml of 5% tri-

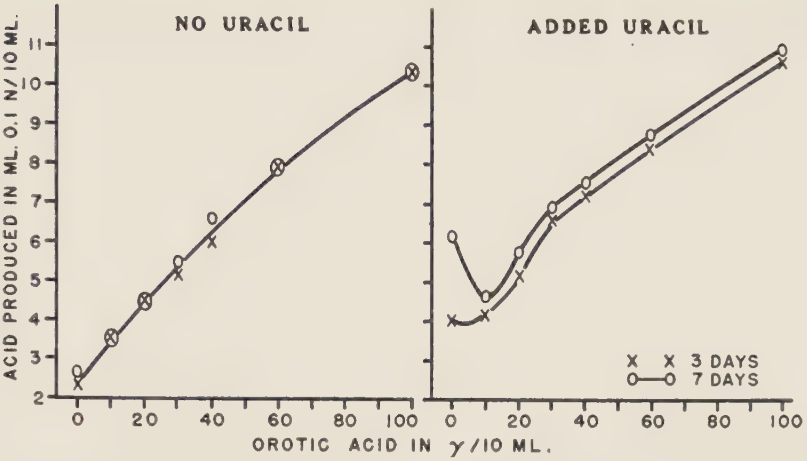

FIG. 1. Response of *Lactobacillus bulgaricus* 09 to orotic acid in the presence and absence of uracil as a component of the basal medium.

chloroacetic acid and the nucleoproteins then were hydrolyzed with 2 ml of 5% trichloroacetic acid at 100°C for 30 minutes. The precipitated proteins were centrifuged and the supernatant solutions containing the liberated nucleic acids hydrolyzed with equal volumes of 2 N HCl at 100°C for one hour. The hydrolysates containing free purines bases and pyrimidine nucleotides were chromatographed on paper using the tertiary butanol-0.8 N HCl mixture of Smith and Markham(15). The pyrimidine nucleotides were located with the aid of a Mineralight lamp and were eluted with water. The water eluates (5 to 10 ml) of each nucleotide were chromatographed on Dowex-1 (Cl^-) columns (about 0.7 cm x 15 cm) according to Cohn(16). Cytidylic acid was eluted with 0.002 N and uridylic acid with 0.01-0.02 N HCl. Those fractions with satisfactory absorption ratios at 278/262 mμ were quantitated in the Beckman spectrophotometer and evaporated to dryness. The molecular extinction coefficients of Ploeser and Loring(17) were used in these calculations. The published molecular extinction coefficients were found to be in good agreement with those found in this laboratory for high quality commercial samples of uridylic and cytidylic acids. The dried purified pyrimidine nucleotides were taken up in small volumes of water and plated. Counts on the labelled compounds and the isolated pyrimidine nucleotides were obtained with a gas flow counter.

Results and discussion. Results of a growth experiment are summarized in Fig. 1. In the absence of uracil from the basal medium growth with or without orotic acid is not influenced by increasing the period of incubation from 3 to 7 days. In the presence of uracil as a component of the basal medium, the growth response to orotic acid is not influenced by prolonging the period of incubation from 3 to 7 days. On the other hand, with uracil growth occurs in the blanks (no added orotic acid) to yield the catenary type curves previously referred to. The mechanism of the selection pressure in favor of those organisms dependent upon orotic acid for growth has not been elucidated.

Results of the isotopically-labelled incorporation experiments are summarized in Table II. It is apparent that if incubation be prolonged (7 days) (Exp. 3) bacterial uridylic and cytidylic acids may be derived from uracil as well as orotic and ureidosuccinic acids (Exp. 1 and 2). These data, together with the results obtained from the growth studies, would appear to indicate the existence in *Lactobacillus bulgaricus* 09 cultures of spontaneously occurring mutants that utilize uracil for purposes of pyrimidine nucleotide synthesis. In the presence of equal amounts of orotic acid and C^{14}-labelled uracil about 5% of the uricylic and cytidylic acids synthesized appear to be derived from uracil. Of special interest is the fact that despite the variety of

TABLE II. Utilization of C^{14}-Uracil for the Synthesis of Uridylic and Cytidylic Acids by *Lactobacillus bulgaricus* 09.

Exp. No.	Pyrimidine source	Incubation, days	Activities: Compound counted	Activities: cpm/μM	Activities: % of pyrimidine source
1	C^{14}-orotic acid*	3	Orotic acid	11650	
			Uridylic "	7500	64
			Cytidylic "	7950	68
2	C^{14}-ureidosuccinic acid*	3	Ureidosuccinic acid	12060	
			Uridylic "	7600	63
			Cytidylic "	7000	58
3	C^{14}-uracil	7	Uracil	14936	
			Uridylic acid	13019	87
			Cytidylic "	11338	76
4	Unlabelled orotic acid C^{14}-uracil	3	Uracil	14936	
			Uridylic acid	1013	6.8
			Cytidylic "	693	4.6

* Data from these experiments have been presented in a preliminary note(6).

pyrimidine sources employed to promote growth and the differences in time required to attain maximal growth (1-2 days with orotic acid (Exp. 1), 2-3 days with ureidosuccinic acid (Exp. 2), and 7 days with uracil alone (Exp. 3)) all of the pyrimidine nucleotides isolated were less active than were the labelled compounds used to promote growth. Some possible explanations for this phenomenon are the following: (a) orotic acid and/or uracil is required in nucleic acid synthesis only as a "primer" to initiate growth; (b) alternative pathways for pyrimidine nucleotide synthesis may exist that do not involve orotic acid, uracil, or ureidosuccinic acid as intermediates; (c) bacterial uridylic and cytidylic acids may be of two "types," one type derivable from known precursors on which *Lactobacillus bulgaricus* 09 is dependent for growth and the other type(s) derivable from other unrecognized components of the basal medium; and (d) orotic acid and/or uracil is required for some essential function quite unrelated to pyrimidine nucleotide synthesis and its incorporation into nucleic acid components is only incidental.

Summary. Growth and isotopically-labelled incorporation studies have shown that *Lactobacillus bulgaricus* 09 utilizes uracil in lieu of orotic acid provided the incubation time is greatly prolonged. Some implications of this finding are discussed.

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Experimental Bacterial Endocarditis in Altitude Rats. III. Effect of Age of Infection on Response to Penicillin. (19802)

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In previous papers(1-3), it was shown that severe cardiac damage could be induced in rats by 4-hour daily exposures in a low-pressure chamber to a simulated altitude of 25,000 feet, and that bacterial endocarditis and renal lesions could be readily produced in such rats by the intravenous injection of bacteria. In this paper, we are reporting on the response of such experimentally-induced endocarditis and renal lesions to penicillin therapy, and the effect of the age of the infection on the therapeutic response. The methods used in exposing the animals to altitude, in inoculating the bacteria, and in preparing the histologic sections, were essentially similar to those described in the previous reports(1-4).

Methods. In the first experiment, 64 young adult white male Holtzmann rats received 70 successive daily exposures to 25,000 feet simulated altitude. The rats, weighing about 250 grams each, were then inoculated intravenously with 0.5 cc of a 6-hour culture of *Streptococcus faecalis* in dextrose blood broth, with a microscopic count of 1 billion bacteria per cc. Treatment was begun 20 hours later. The animals, except those used as controls, were given 200 mg/kg of sodium penicillin G in aqueous solution 6 times daily at 3, 9, and 12 a.m., 3, 6, and 11 p.m. At the intervals indicated in Table I, rats were killed for bacteriologic and histopathologic studies. For the bacteriologic studies, the hearts and kidneys were removed aseptically, emulsified in 50 cc of 1% dextrose blood broth in a Waring blendor for 1½ minutes, and 1 cc of the emulsion was subcultured in dextrose blood

TABLE I. Exp. 1. Effect of Sodium Penicillin G (200 mg/kg 6 Times Daily Intramusc.) on Bacterial Counts* in Hearts and Kidneys of Rats Inoculated 20 Hr Previously with a Single Intrav. Inj. of *Streptococcus faecalis*.

	Days after inoculation	1	2	3	5
	Days after treatment	0	1	2	4
Heart	Untreated controls	2160	606	182400	1760
		108	460	10600	1680
		78	192	120	0
		22			
		6			
		4.7			
	Treatment instituted 20 hr after inoc.		.35	1096	122
			.2	0	.05
			.1	0	.05
			.05	0	0
			0	0	0
Kidney	Untreated controls	16300	131200	141000	550000
		16100	9600	83000	277000
		16600	4800	38000	237000
		10400			
		7300			
		1300			
	Treatment instituted 20 hr after inoc.		434	26	
			62	10.3	508
			20	9.4	1.7
			14	4.6	.5
			0	2.4	.45
					0

* Numbers in the table are total numbers of streptococci in thousands. 0 = <20 organisms in the organ emulsion.

TABLE II. Exp. 2. Effect of Intramusc. Treatment with Sodium Penicillin G* on Bacterial Counts† in Hearts of Rats Inoculated with *S. faecalis.*

Days after inoc.	½	1½	2½	4½	5½	6½	8½	10½
Untreated controls	246	430	43000	48000		146000	7500	
	72	320	252	3200		140000	1.1	
	51	210	190	128		2100	.2	
	30	13	4.1	120		.7		
	28	2.9	.7	78		0		
	13			.7				
	6.8							
Treatment instituted early, 12 hr after inoc.		12	106	.57		.17		
		2.9	.033	.47		.17		
	Penicillin	3.1	.033	0		0		
	begun	2.2	0	0		0		
		1	0	0		0		
		.5	0	0		0		
Treatment instituted late, 4½ days after inoc.					67000	90000	.033	1950
					61000	25000	0	.033
			Penicillin begun		28.7	.67	0	.03
					1.5	.67	0	0
						<.6	0	
							0	

* Treatment at 200 mg/kg 4 times daily plus 20 mg/kg "Benemid" twice daily.

† Numbers in table represent total bacterial count in thousands. 0 = <20 organisms in the total heart emulsion.

TABLE III. Exp. 2. Effect of Intramuscular Treatment with Sodium Penicillin G* on Bacterial Counts† in Kidneys of Rats Inoculated with *S. faecalis.*

Days after inoc.	½	1½	2½	4½	5½	6½	8½	10½
				× 1000				
Untreated controls	2.85	1430	406	2050		2600	4142	
	1.60	66	137	1500		2400	1238	
	.91	53	32	1370		2050	1070	
	.85	33	5.7	642		1370		
	.67	11	4.3	534		934		
	.61			192				
	.35							
Treatment instituted early, 12 hr after inoc.		750	400	1200		3000000		
		501	47	1.9		86.4		
	Penicillin	320	42	.95		40.5		
	begun	124	31	.77		1.23		
		64	3.7	.39		0		
		33	1.7	0		0		
						× 1000		
Treatment instituted late, 4½ days after inoc.					693	2600	255	107
					420	2396	173	107
			Penicillin begun		353	1505	4	55
					73	453	0	1.9
						406	0	
							0	

* Treatment at 200 mg/kg 4 times daily plus 20 mg/kg "Benemid" twice daily.

† Numbers in table represent total bacterial count in thousands. 0 = <20 organisms in the total kidney emulsion.

It is to be noted that the numbers in the top and bottom sections are to be multiplied by 1000 to give the count in thousands.

agar. In a second experiment, 183 young adult white male Holtzmann rats received 30 successive daily exposures to altitude and then were inoculated intravenously with 0.5 cc of a 4-hour streptococcal culture. The inoculum contained 1150 x 10^6 organisms/cc by microscopic count, and 840 x 10^6 by plate count. The animals were then divided into 3

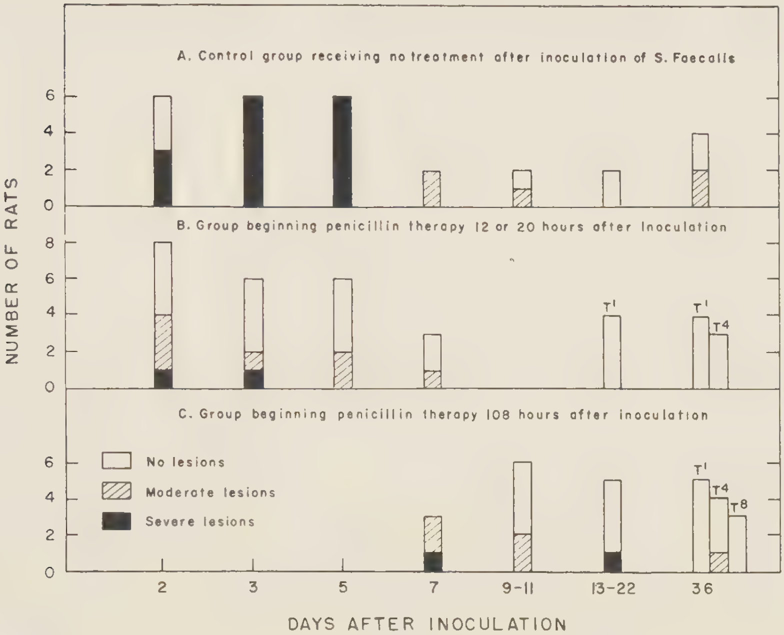

FIG. 1. Effect of penicillin therapy on development of cardiac lesions in altitude rats inoculated with *Streptococcus faecalis*. All rats in groups (B) and (C) were treated until end of specified period except those in columns T^1, T^4, and T^8, which were treated for only 1, 4, and 8 days respectively. In columns marked 13-22, rats in groups (A) and (C) were killed at 13 to 16 days after inoculation and rats in group (B) at 22 days. No spontaneous deaths are included.

groups. Two groups were treated intramuscularly 4 times daily (9:00 a.m., 1:00 p.m., 5:00 p.m., 11:00 p.m.) with sodium penicillin G at 200 mg/kg and twice daily with "Benemid"* at 20 mg/kg. Treatment of the first group was begun at 12 hours and of the second group at 4½ days after inoculation. The third group served as an untreated control. Bacteriologic studies were made as described in the first experiment. In the control, untreated animals the bacterial counts in the blood at autopsy (1 cc samples) varied from 0 to 244 per cc, numbers which were insignificant in relation to those found in the heart and kidney. In all but one of the treated animals, the bacterial count in the blood at autopsy was less than 23 per cc; and in more than two-thirds of the treated animals, duplicate blood cultures on 1 cc samples were negative, including animals with massive bacterial involvement of the heart and kidney.

Results. a) *Bacteriologic studies.* As shown in Tables I, II, and III, in both the heart and kidney, early treatment, begun either 12 or 20 hours after inoculation usually effected a significant and fairly rapid reduction in the bacterial count, as contrasted with the rapid and progressive increase in the control untreated animals. However, when treatment was delayed until 4½ days after inoculation, the effect of penicillin on the bacterial count in the kidney was not apparent until the 8th day, 4 days after the beginning of treatment. The effect of delayed treatment on the bacterial count in the heart was obscured by the variability of the results obtained in both the control and treated animals.

b) *Pathology.* The histopathologic findings in the 2 experiments are summarized in Fig. 1 and 2. The lesions were graded severe (black in the columns) only if bacteria were noted in the microscopic sections. In the heart, streptococci occurred in large masses in the valvular vegetations and in the kidneys they were usually found in necrotic lesions in the renal papillae. In the kidneys there were also scattered inflammatory foci of variable size, in which were tubules contain-

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* Furnished by Sharp and Dohme.

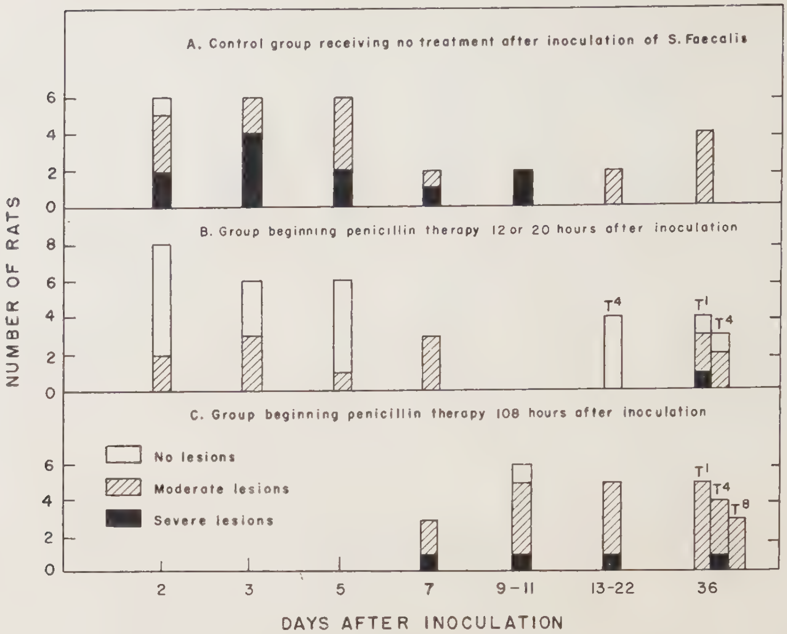

FIG. 2. Effect of penicillin therapy on development of renal lesions in altitude rats inoculated with *Streptococcus faecalis*. All rats in groups (B) and (C) were treated until end of specified period except those in columns T^1, T^4, and T^8, which were treated for only 1, 4, and 8 days respectively. In columns marked 13-22, rats in groups (A) and (C) were killed at 13 to 16 days after inoculation and rats in group (B) at 22 days. No spontaneous deaths are included.

ing a purulent exudate occasionally admixed with bacteria. Lesions were graded as moderate if there were inflammatory changes without evident bacteria. These represent mild, early developing, or late regressing lesions. In the heart, regression or healing of severe lesions was suggested by the occurrence of large organizing vegetations, in which there were no visible bacteria and relatively few polymorphonuclear leucocytes. In the kidney, regression was evidenced by depressed scars infiltrated by lymphoid cells, with a purulent exudate in a relatively small number of tubules. Calcification was sometimes noted in a few scattered tubules, and occasionally there was apparent sloughing into the pelvis of the necrotic portion of a renal papilla.

The 12-hour and 20-hour treatment groups have been combined in the figures since in both, the early treatment appeared to have been effective in suppressing the development of serious cardiac or renal lesions during treatment. This agrees with the rapid disappearance of bacteria from these organs (Tables I, II, III). Treatment was perhaps more effective in the 12-hour group, since only 1 of 11 rats in this group examined immediately after treatment for 1 to 4 days showed lesions, consisting of a sterile vegetation and a focal leucocytic infiltration of the mitral valve. In contrast, 7 of 9 similarly treated rats in the 20-hour group had moderate to severe valvular lesions and 6 had moderate suppurative renal lesions. None of the 11 rats treated 12 hours after the inoculation and killed for histologic study after penicillin treatment for 1 to 4 days had significant renal lesions. However, 4 to 5 weeks later, 5 of 7 animals in this group (Fig. 2, group B) had regressing suppurative renal lesions as described above. These findings are consistent with the fact that such early treatment usually suppressed the multiplication of bacteria, but had sterilized the kidneys in only 3 of 12 rats examined immediately after treatment for 4 to 6 days. It is evident, therefore, that treatment for 4 to 6 days may temporarily suppress the kidney infection, but often does not prevent subsequent bacterial multiplication and the development of lesions in this group.

In the animals in which treatment was not begun until 4½ days after inoculation, treatment had little effect on the renal lesions, which by that time ranged from moderate to severe. This agrees with the fact that the bacterial count in the kidneys also was not immediately affected in this group.

The effect of delayed treatment on the cardiac lesions is difficult to evaluate because of the small number of controls, and because the incidence and severity of cardiac lesions were relatively low in animals which survived without treatment (Fig. 1). The lesions tended to be more severe in animals that died than in those that were killed for study at corresponding periods. These spontaneous deaths have not been included in the tables or figures.

c) *Mortality.* The cumulative mortality† in untreated rats during the first 4½ days following inoculation was 8%. In the following 5 weeks, the cumulative mortality in the surviving untreated rats (controls) was 55%. Over the same period, in rats beginning treatment 4½ days after inoculation, the cumulative mortality was 17%. In animals treated 12 hours after inoculation, the cumulative mortality was 2%, one of 51 rats dying 8 hours after the beginning of treatment.

(d) *Discussion.* The contrasting effects of early and late treatment on the bacterial count, on the histopathologic findings in the heart and kidney, and on the mortality, agree qualitatively with the results previously reported in a group A streptococcal infection in mice(5). Both infections were easily controlled by early treatment with penicillin, but were relatively refractory to treatment instituted later, after there had been marked bacterial proliferation. These findings may have a bearing on the known refractoriness of bacterial endocarditis to treatment with penicillin. Possible explanations have been discussed in the previous paper(5).

Summary and conclusions. Rats were exposed daily to simulated high altitude and then inoculated intravenously with a young broth culture of *Streptococcus faecalis.* (a) Untreated rats developed a vegetative bacterial endocarditis and renal abscesses and necrosis. (b) Treatment with penicillin instituted within 12 or 20 hours after inoculation was highly effective in reducing the mortality, and in aborting or preventing the development of cardiac lesions. In the kidney, such early treatment effected an immediate marked reduction in the average bacterial count and in the incidence and severity of lesions. However, 4-5 weeks after the cessation of treatment renal lesions were noted in 5 of 7 animals, presumably caused by surviving bacteria. (c) When treatment was delayed until 4½ days after inoculation, permitting extensive bacterial proliferation and the development of pathological changes, penicillin therapy had a much less favorable effect on mortality, bacterial counts, and the regression of lesions.

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† Calculated for each experimental group by a modified life table technic. Mortality rate was not computed for rats treated 20 hours after inoculation since nearly all were killed within 5 days.

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Oxygen Consumption Following Whole-Body X-Irradiation of Guinea Pigs.
(19803)

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By means of direct measurements of oxygen consumption in rats, increased Qo_2 has been reported to follow doses of total body X-irradiation ranging from 54 r to over 800 r(1). Smith *et al.*(2) could find no increased oxygen consumption in irradiated rats, but were able to detect a small increase in oxygen consumption of starved irradiated rats over their starved controls. The present report indicates that no significant change in oxygen consumption occurs after an LD_{30} dose of X-rays to guinea pigs.

Experimental. Oxygen consumption was determined in Exp. 1 by means of the apparatus described by Smith(3), while in Exp. 2 a modification of the instrument described by Tabor *et al.*(4) was used. The guinea pigs in Exp. 1 were fasted for 4 hours and those in Exp. 2 (Table I) for 20 to 24 hours prior to the measurement of oxygen consumption. With the animal in the chamber, 30 minutes were allowed for temperature equilibration and the Qo_2 for each observation was determined from the oxygen consumed during the succeeding 30 minutes. Twelve controls and their corresponding irradiated litter mates were tested for oxygen consumption on alternate days in each experiment. Guinea pigs of the Beltsville strain were given 250 r, a dose which was about an LD_{30} since 4 of 12 had died by 11 days after irradiation. Irradiation factors were: 170 Kv, 20 ma., 0.25 mm copper and 0.55 mm aluminum added filtration, 50 cm focal distance and rate 54 r per minute.

TABLE I.
Comparison of Mean Oxygen Consumption in Guinea Pigs Given 250 r of X-rays on Day R with Oxygen Consumption in Their Litter Mate Controls.

Days	No. observations	Mean Qo_2, ml/g/hr S.T.P.	Stand. dev.
Exp. 1			
	Controls		
R — 3 to R +29	58	1.298	±.29
	250 r at day R		
R — 4 to R — 1	16	1.366	±.17
R + 1 R + 3	12	1.254	±.29
R + 4 R + 6	8	1.504	±.26
R + 7 R + 9	11	.969	±.17
R +10 R +13	10	1.273	±.36
Exp. 2			
	Controls		
R — 7 to R +24	60	.757	±.11
	250 r at day R		
R — 7 to R — 1	15	.808	±.03
R + 1 R + 3	11	.733	±.06
R + 4 R + 6	7	.726	±.05
R + 7 R + 9	9	.811	±.31
R +10 R +13	9	.731	±.11

Results and discussion. Oxygen consumption expressed in ml O_2 (S.T.P.) per hour per g of body weight for guinea pigs and their irradiated litter mates as found in 2 experiments is given in Table I. Relatively high values for oxygen consumption obtained in the first experiment are attributable to the short fast period (4 hours) which preceded the tests. The longer period of fast (20-24 hours) for the animals used in Exp. 2 resulted in oxygen consumption values which are comparable with basal Qo_2's reported for guinea pigs(5). No significant change in oxygen consumption up to 13 days following 250 r is indicated from the data given in Table I. Irradiated guinea pigs given 250 r gain weight at the same rate as their litter mate controls and Fig. 1 compares the weights of a typical lot of 4 guinea pigs from Exp. 1 with their irradiated litter mates. Guinea pigs given comparable doses of irradiation were shown by Smith *et al.*(6) to gain weight normally. The absence of altered oxygen consumption during the period of tissue destruction and regeneration following irradiation, in the guinea pigs, indicates that within the limits of the experiment no detectable disturbance to the overall metabolism occurred.

Summary. Oxygen consumption in guinea pigs given 250 r, remains within the limits de-

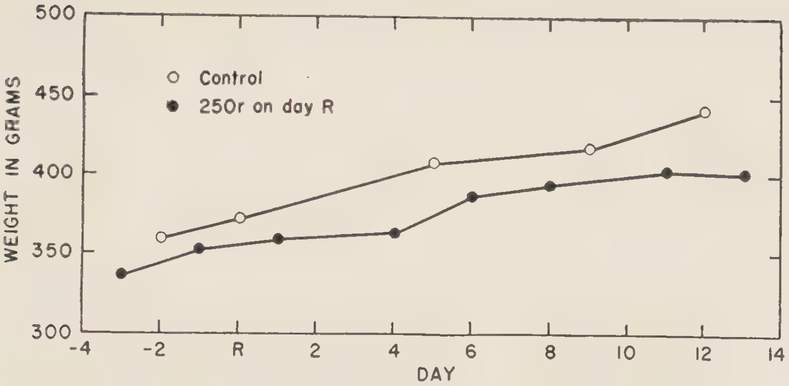

FIG. 1. Comparison of the mean weights in a typical lot of 4 guinea pigs from Exp. 1 with their corresponding litter mates given 250 r on day R.

termined for their nonirradiated litter mates up to 13 days following irradiation.

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Inotropic Action of Acetyl-Strophanthidin, Strophanthidin, and Tryptamine-Strophanthidin.* (19804)

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The partially synthetic ester, acetyl-strophanthidin, which has an extremely rapid onset of action after intravenous injection, has been suggested for use when digitalis action is urgently needed(1). It has proved valuable for slowing the ventricular rate in patients with auricular fibrillation, and for revision of paroxysmal auricular tachycardia(1,2). In pulmonary edema with normal sinus rhythm, where it has also been used, its value cannot be proven, for an experiment withholding other therapeutic measures would risk the life of the patient. Recently the clinical use of acetyl-strophanthidin has been challenged by Wedd and Blair. In strips of turtle ventricle they found that it failed to shorten the interval between action potentials (interpreted as the Q-T interval) without impairing contractility(3). Since digitalis glycosides do shorten the "Q-T interval" of turtle ventricle(4), Wedd and Blair concluded that acetyl-strophanthidin is not a digitalis-like substance, and that all its therapeutic benefit is due to vagal stimulation.

If acetyl-strophanthidin does not have a typical digitalis action, its use in patients with pulmonary edema and sinus rhythm should be discontinued. A reexamination of acetyl-

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TABLE I. Effect of Strophanthidin Derivatives on Force of Contraction of Hypodynamic Myocardium.

			Drug response	
Drug	No. of muscles	Avg hypodynamic contraction (% of initial force)	Conc. (molar × 10^{-7})	Avg improvement in contraction (% of hypodynamic force)
Acetyl-strophanthidin	7	20	2.25	355
			4.5	770
Strophanthidin	3	28	4.5	212
			9	301
			13.5	381
Tryptamine-strophanthidin	4	19	4.5	333
			9	471

strophanthidin was indicated, with special attention to its effect on the force of contraction of failing myocardium free of nervous influence. For this purpose we utilized the papillary muscle from the right ventricle of the cat heart, as described by Cattell and Gold(5). In addition to acetyl-strophanthidin, strophanthidin, and tryptamine-strophanthidin were studied.[†]

Method. Details of the method may be found in a recent publication by Garb and Chenoweth(6). Suitable papillary muscles were rapidly excised, immersed in oxygenated Krebs-Ringer phosphate, and stimulated electrically at the rate of 40 contractions per minute. The cordi tendeni were tied to a tension lever with a mirror attached, and the contractile force recorded by the deflection of a light beam on a fixed scale. When the force of contraction had failed to about 25% its initial value, one of the compounds was added to the solution bathing the muscle, and the force measured at intervals for a period of hours. In many experiments the concentration of drug was increased after the force of contraction had reached a steady state. The concentration of each substance was chosen to approximate the value expected in the blood of human patients following intravenous injection of a therapeutic dose.

Results. Each of 14 hypodynamic papillary muscles developed an increase in force of contraction following one of the strophanthidin derivatives. The results are summarized in Table I. The inotropic response of each muscle lasted throughout the period of observation of from 2 to 8 hours, average duration 4.3 hours. Molecule for molecule, acetyl-strophanthidin was more potent than the other 2 compounds. Occasional extrasystoles were noted in one muscle which received acetyl-strophanthidin and one which received tryptamine-strophanthidin. In Fig. 1, acetyl-strophanthidin induced an inotropic response which persisted until the experiment was discontinued 6 hours later. The response of the muscle that received strophanthidin illustrates the common trend in which increased force of contraction follows each increase in concentration. The final concentration of strophanthidin in Fig. 1 (2.7 x 10^{-6} M) produced the typical sharp rise and rapid decline in force of contraction seen as the toxic effect of digitalis glycosides(7). There were also extrasystoles, increase in resting tension, and increase in the threshold of excitability. Another muscle, placed in 9.0 x 10^{-7} M acetyl-strophanthidin, showed similar toxic effects.

Discussion. The direct myocardial action of digitalis was first demonstrated by Cattell and Gold(5) with the isolated papillary muscle preparation. They observed that digitalis increases the force of contraction of failing mammalian heart muscle. Later, Gold and Cattell(8) concluded that this direct action is the primary therapeutic mechanism of digitalis. Their conclusion has aroused controversy, but no substantial evidence has weakened it; the data available in patients with heart disease, congestive failure, and sinus rhythm(9,10) are best interpreted by accepting the view that increased force of contraction is the mechanism of digitalis effect. Despite the agreement of laboratory and clinical evidence, there are no universally accepted criteria which distinguish a drug as

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[†] These compounds were supplied through the courtesy of Dr. K. K. Chen of Eli Lilly and Co.

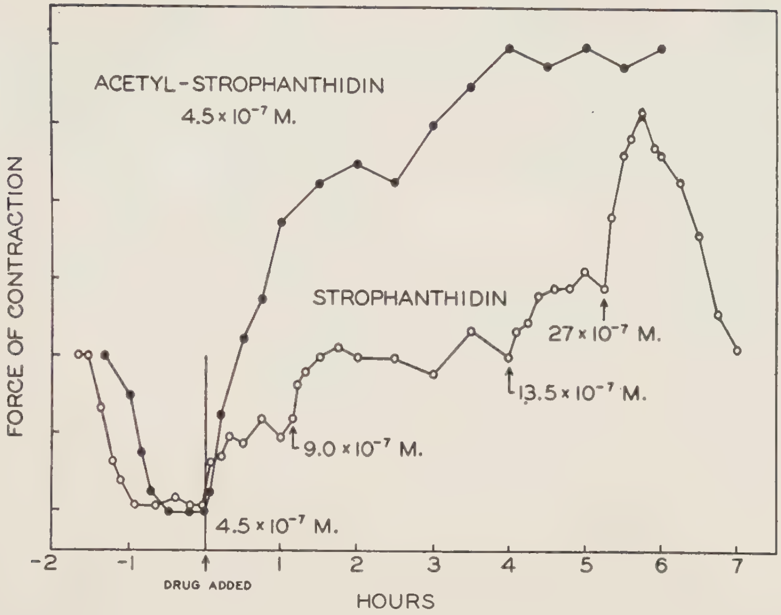

FIG. 1. Force of contraction of 2 representative papillary muscles illustrating the hypodynamic state, and the inotropic response to strophanthidin derivatives. The concentration of drug is given in molarity of the fluid bathing the muscle. The responses are similar to those from digitalis glycosides.

having the effect of digitalis. Changes in action potential, such as those described in strips of turtle ventricle, are sometimes used to characterize digitalis-like drugs. It should be noted that the relation between electrical activity and the therapeutic action of digitalis is not clear. Furthermore, the calcium ion, long associated with digitalis effect, is known to influence action potential and contractile force independently(11).

The effect on force of contraction might well be the critical issue in determining whether a drug is to be considered similar to digitalis, and for resolving that question, the isolated hypodynamic papillary muscle is the most convenient laboratory preparation. The concentration of drug in its bath fluid must be in the range possible after intravenous injection of digitalizing doses in human patients (the dose determined by slowing of the ventricular rate in patients with auricular fibrillation). As pointed out by Gold(12), with the proper concentration of the drug, only digitalis yields an improvement in contractile force of the papillary muscle which persists for many hours. The ester, acetyl-strophanthidin, the genin, strophanthidin, and the tryptamine derivative of strophanthidin increased the force of contraction of failing myocardium, and did so in concentrations provided by intravenous injections in patients with auricular fibrillation(1,13,14). Thus they meet our criteria for a digitalis-like substance. Acetyl-strophanthidin was the most potent of the 3 on the papillary muscle, just as it is in human patients.

Previous clinical investigations have established the value of acetyl-strophanthidin for the control of acute congestive failure with auricular fibrillation, and for the revision of paroxysmal auricular tachycardia(1,2). Its special advantage lies in facilitating rapid digitalization by the common scheme, in which a safe dose is given first, and then additional small doses are given until the therapeutic response or minor toxic symptoms appear. In order to avoid serious toxicity, additional doses must be delayed until the full effect of the previous doses has developed. The rapid onset of action of acetyl-strophanthidin reduces the interval between doses to 10 minutes (as compared to one hour for ouabain), and provides the fastest digitalization possible with preparations now in use(1). The present demonstration of its positive inotropic action supports the continued use of acetyl-strophanthidin as one of the emergency measures in the treatment of acute congestive failure with pulmonary edema in the presence of normal sinus rhythm.

Summary and conclusions. 1. Papillary muscles from the right ventricle of the cat heart were stimulated in phosphate-buffered Krebs-Ringer solution until their force of contraction declined. 2. On addition of acetyl-strophanthidin, tryptamine-strophanthidin, or strophanthidin to the papillary muscles, their force of contraction improved, and the improvement persisted throughout the period of observation (average 4.3 hours). 3. It is concluded that these strophanthidin derivatives have a typical digitalis action on failing mammalian heart muscle.

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Poliomyelitis Viruses in Tissue Culture. III. Susceptibility of Testicular Tissue from Immune Monkeys.* (19805)

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Tissue culture technics have been applied in the study of poliomyelitis to the propagation of laboratory-adapted strains of the virus (1-8), to the quantitative estimation of virus and antibody(9-11), and to the isolation and immunologic identification of poliomyelitis viruses from various sources(12-14). The use of monkey testicular tissue by some investigators for these purposes raises a question of both theoretical and practical importance, *i.e.*, whether poliomyelitis viruses may be propagated in cultures of testicular tissue obtained from monkeys immune to these viruses. In an attempt to answer this, a comparison was made of the susceptibility of testicular tissue obtained from both immune and non-immune monkeys to poliomyelitis viruses. Observations of this nature also bear on the use of human tissues for the cultivation of poliomyelitis virus, for these are usually obtained from individuals whose immune status is unknown.

Materials and methods. Tissue cultures. Roller tube cultures were employed in these

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[†] Sheffield Fellow of Yale University 1950-1952. The data presented in this paper form part of a thesis presented by N. L. to the Graduate School of Yale University in partial fulfillment for the degree of Doctor of Philosophy.

experiments. The technic and the nutrient medium used have been described in detail (11). Testes from immature rhesus (*Macaca mulatta* and cynomolgus (*Macaca irus*) monkeys were used as the source of tissue, for testes from both species have been found to be equally susceptible to poliomyelitis virus in roller tube tissue cultures(15). *Viruses.* These were used in the form of tissue culture fluid pools: (a) Y-SK (type 2) strain in its 14th passage in monkey testicular culture, having a tissue culture endpoint of 10^{-2}; (b) Leon (type 3) strain in its 8th culture passage, its titer being 10^{-2}, (c) Brunhilde (type 1) strain in its 9th culture passage, its titer being 10^{-3}. Virus titrations were performed using serial 10-fold dilutions. In these experiments, the tissue culture endpoint is the final dilution of virus producing unmistakable tissue degeneration in 4 days (11). *Antisera.* These were obtained from monkeys immunized to the 3 types of poliomyelitis virus: Brunhilde, Y-SK, and Leon. A 20% suspension of monkey cord was made, centrifuged at 2000 RPM for 10 minutes, and mixed with adjuvant(19) in the following proportions: 2 ml of Arlacel, 8 ml of Bayol F, and 10 ml of virus. Three weekly injections of 2 ml of the mixture were given intramuscularly. The monkeys were bled 30 days after the last injection. *Neutralization tests in tissue culture.* Various dilutions of unheated sera were mixed with a final amount of 100 tissue culture doses of virus, and the mixture incubated for one hour at room temperature. Neutralization titers were determined at the end of 4 days as described(11).

Experimental. Before attempting to determine whether multiplication of poliomyelitis viruses may occur in testicular tissue obtained from immune monkeys, it was necessary to determine the degree of susceptibility of tissue obtained from different non-immune monkeys. In 2 different experiments, testes were removed from a total of 10 "normal" monkeys, and roller tube cultures were prepared separately from the tissue of each. Serial 10-fold dilutions of the Y-SK virus pool were inoculated into a series of roller tube cultures prepared from the testes of each monkey. The tissue culture endpoint was read on the fourth day. At the end of 8 days, at which time virus growth reaches its maximum(11), the viral concentration of the fluid from the tubes inoculated with undiluted virus was determined by titration in roller tubes.

TABLE I. Susceptibility of Cultures of Testicular Tissue Obtained from 10 Different Monkeys to the Y-SK Strain of Poliomyelitis Virus.

		Titration endpoints at 4 days	
	Monkey No.	Tissue culture endpoint of Y-SK	Virus recovery from tubes 8 days after inoculation with undiluted tissue culture virus
Exp. 1	Rh 5640	10^{-2}	100†
	5641	10^{-2}	100
	5642*	10^{-1}	10
	5643	10^{-2}	100
	5644	10^{0}	1
Exp. 2	Rh 5646	10^{-2}	100
	5647		
	5648		
	5649		
	5650		

* Monkey 5642 testicular tissue showed poor growth in culture.

† 100 tissue culture doses were recovered per .1 ml, *i.e.*, this fluid gave a titer of 10^{-2} on the 4th day in a new series of tubes.

From the results listed in Table I, it is evident that the titration endpoint of Y-SK virus was uniformly reproducible in the individual tissues of 8 out of the 10 monkeys tested. The tissue of monkey 5642, in contrast to the others tested under the same conditions, showed poor growth in culture, and yielded a decreased amount of virus. The tissue of monkey 5644 definitely gave a decreased titer using the cytopathic endpoint, and the yield of virus was also low. Since this finding could not be explained by the poor growth of the tissue, the question arose whether the possible presence of antibody may have been the cause. Therefore, experiments were next designed in which the serum of each monkey whose tissue was to be tested for susceptibility to the Y-SK virus was also examined for the presence of antibody.

In 2 separate experiments, testes were removed from a total of 8 "normal" monkeys, and roller tube cultures were prepared separately from the tissue of each monkey. Serial

TABLE II. Susceptibility of Cultures of Testicular Tissue Obtained from 8 Different Monkeys to the Y-SK Strain of Poliomyelitis Virus.

	Monkey No.	Tissue culture endpoint at 4 days	Serum antibody
Exp. 1	Rh 6095	10^{-2}	0
	6097	10^{-2}	0
	6101	10^{-2}	0
	6103	10^{-3}	0
Exp. 2	Rh 5115	10^{-2}	0
	5116	10^{-2}	0
	Cyno 5796	10^{0}	0
	5798	10^{-2}	0

10-fold dilutions of the same Y-SK virus pool, as used in the previous experiments, were inoculated into a series of tubes prepared from the testes of each monkey. The tissue culture endpoint was read in 4 days. At the time of removal of the testes, the monkeys were bled. Undiluted serum was tested for neutralizing activity against Y-SK virus. The results are given in Table II. Of the 8 monkeys tested for susceptibility to the Y-SK virus, 6 showed a tissue culture endpoint of 10^{-2}. The tissue of Cyno 5796 grew well but it poorly supported virus growth. Since this monkey had no serum antibody at the time the tissue was removed, other factors must be considered responsible. Whether the tissue of Rh 6103, which gave an endpoint of the virus of 10^{-3}, represents an especially susceptible monkey is open to speculation. These experiments are representative of a large series of separate experiments.

Experiments were then planned to determine whether testicular tissue obtained from immune monkeys would support the growth of poliomyelitis viruses. A series of immune monkeys were selected and tested for their susceptibility to the viruses with which they had been immunized, and in certain instances to a heterotypic virus as well. Four experiments were designed:

Exp. 1. Testes were removed from 4 Y-SK immune monkeys, and roller tubes were prepared separately. Serial 10-fold dilutions of Y-SK virus were added to 5-day growing tissue, and the endpoint was read in 4 days. The sera of the monkeys were tested with the same virus, Y-SK, using the following final serum dilutions: 1:10, 1:100, 1:1000, and 1:10000.

Exp. 2. In the regular procedure employed in making roller tubes the tissue is washed twice before being used. The washing was intentionally omitted in the experiment. The tissue was minced, suspended in 5 ml of salt solution, and roller tube cultures immediately prepared. In addition to allowing the tissue to incubate for 5 days before inoculating the virus, dilutions of the virus were added to the tubes at 0 time of incubation. Considerable dilution of the antibody might take place in 5 days, due to 2 renewals of the nutrient medium before virus inoculation in the regular procedure(11). The tubes inoculated at 0 time of incubation were read 9 days after inoculation of virus, at the same time when the tubes, inoculated after 5 days of tissue incubation, were examined for degeneration 4 days after inoculation of the virus. The tissues of 5 Y-SK immune monkeys were tested, and the same strain was used in testing their susceptibility in culture and in the neutralization tests.

Exp. 3. Two Leon immune monkeys were used, and their testicular tissue and sera were tested with the homologous strain.

Exp. 4. The testicular tissue and sera of 3 Brunhilde immune, and 2 Y-SK immune monkeys were tested with both Brunhilde and Y-SK viruses, according to the procedure of Exp. 1.

The results are given in Table III. The testicular tissues of 9 Y-SK immune monkeys, whose serum antibody titers ranged from 1:10 to 1:1000, were tested for susceptibility to the Y-SK virus. In all cases, the tissue culture endpoint of the virus was found to be 10^{-2}, the titer which was obtained in tissue from non-immune monkeys. Even though the regular procedure of washing the tissue before the preparation of roller tubes was omitted and the tubes inoculated immediately with virus, the tissue culture endpoint was still found to be 10^{-2}. Of the 2 Leon-immune monkeys tested, the testicular tissue of both proved equally susceptible to the action of the virus. The tissue culture endpoint was found to be 10^{-2}, the titer obtained in tissues from non-

TABLE III. Susceptibility of Testicular Tissue of Different Immune Monkeys to Poliomyelitis Virus.

Monkey No.	Immune to:	Tested vs.:	T.C. endpoint of virus at 4 days — Tissue inoc. at — 0 days	5 days	Serum neutralization titer in T.C.*
Exp. 1					
Cyno 5255	Y-SK	Y-SK		10^{-2}	1:1000
5464				10^{-2}	1:1000
5806				10^{-2}	1:10
5728				10^{-2}	1:100
Exp. 2†					
Rh 6043	Y-SK	Y-SK	10^{-2}‡	10^{-2}	1:10
6045			10^{-2}	10^{-2}	1:10
6048			10^{-2}	10^{-2}	1:10
6049			10^{-2}	10^{-2}	1:10
6050			10^{-2}	10^{-2}	1:10
Exp. 3					
Rh 6052	Leon	Leon		10^{-2}	1:1000
6054				10^{-2}	1:1000
Exp. 4					
Rh 6055	Brunhilde	Brunhilde		10^{0}	1:100
		Y-SK		10^{0}	0
6056	”	Brunhilde		10^{-3}	1:1000
		Y-SK		10^{-2}	0
6057	”	Brunhilde		10^{-3}	1:1000
		Y-SK		10^{-2}	0
6042	Y-SK	Brunhilde		10^{-3}	0
		Y-SK		10^{-2}	1:10
6044	”	Brunhilde		10^{-3}	0
		Y-SK		10^{-2}	1:10

* The serum neutralization titer was determined with the virus used to obtain the tissue culture endpoint.

† Tissue was not washed as in the routine procedure.

‡ Tissue culture endpoint of virus read at 9 days.

immune monkeys. The serum antibody titer of both monkeys was 1:1000.

In Exp. 4 in Table III, the testicular tissues of 3 Brunhilde immune monkeys and 2 Y-SK immune monkeys were tested for susceptibility to both the Brunhilde and Y-SK poliomyelitis viruses. While the tissue of 2 of the Brunhilde-immune monkeys gave a titer of 10^{-3} with the Brunhilde strain, the tissue of the third Brunhilde-immune monkey, Rh 6055, proved to be "incompletely" susceptible to this virus. The tissue culture endpoint obtained was only 10°. That this result was not due to the presence of Brunhilde antibodies is indicated by the fact that the tissue culture endpoint obtained with the Y-SK virus was also 10°. The tissue of the other 2 Brunhilde-immune monkeys gave a tissue culture endpoint of 10^{-2} with Y-SK. In like fashion, 2 Y-SK-immune monkeys, with no Brunhilde antibodies, gave a titer of 10^{-3}, and of 10^{-2}, with the Brunhilde and Y-SK viruses, respectively. The serum antibody titers of the above monkeys given in Table III, indicate the specificity of the antibody response.

Discussion. It is apparent from these experiments that the presence of antibody in the serum of a monkey, whose testicular tissue is used for setting up roller tube cultures, plays little part in determining the susceptibility, or resistance, of that tissue to poliomyelitis virus. Testicular tissue obtained from immune monkeys supported growth of poliomyelitis viruses equally as well as tissue obtained from non-immune monkeys, under the experimental conditions used. Other investigators have also found that growth of virus in tissue culture may occur in tissues taken from an immune host. Andrewes found that herpes virus(16) and virus III(17) grew in testes obtained from immune rabbits. Growth of herpes virus occurred, even with no preliminary washing of the tissue. In the experiments reported above, growth of Y-SK (type 2) poliomyelitis virus also occurred in testicular tissue which had received no preliminary washing. However, each of the sera in the last mentioned experiment had a low antibody titer of 1:10. Had the titer been high, virus propagation might have been inhibited.

The testicular tissue obtained from different monkeys possesses fairly uniform susceptibility to infection with the poliomyelitis viruses. Of a total of 34 monkeys tested above, both immune and non-immune, the tissue of only 3 has proved to be "incompletely" susceptible. The cause of the "incomplete" susceptibility of certain monkey testicular tissues is not understood. A nutritional deficiency of the monkey may contribute to this state. The possible presence of an interfering, latent virus should also be considered, and stock monkeys are known to be infected at times with B virus. Last year, a B-like virus was actually isolated from a rhesus monkey in the Yale colony(18). Or cellular susceptibility or insusceptibility may be a genetically controlled character. An "incompletely" susceptible state may be a contributing factor in the failure to propagate poliomyelitis virus in tissue culture, if tissue from a single insusceptible monkey is used. If virus isolations from various sources are to be made, a pool of tissue from more than one monkey is desirable. Such a factor may also influence experimental conclusions dealing with the comparison of tissue culture and animal pathogenicity of various poliomyelitis strains, if tissue from a single monkey is used.

Summary. The titration endpoints of Brunhilde (type 1), Y-SK (type 2), and Leon (type 3) poliomyelitis viruses were uniformly reproducible in roller tube cultures of testicular tissue from 31 of the 34 monkeys tested. The tissue of approximately 10% of the monkeys tested was "incompletely" susceptible to the poliomyelitis viruses. This insusceptible state could not be attributed to the presence of serum antibody. Testicular tissue obtained from immune monkeys supported growth of all 3 antigenic types of poliomyelitis virus comparable to that obtained in tissue of non-immune monkeys.

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Thiocyanate Effect on Sodium Excretion in Normotensive and Hypertensive Subjects.* (19806)

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Although a half-century has passed since the introduction of thiocyanate into the treatment of hypertensive vascular disease, little is known about the mechanism of its depressor action. Effects on cell permeability(1), a parathyroid-like action upon the kidney(2), the production of changes in the adrenal cortex (3) and inhibition of amino-acid oxidation (3a) have been suggested. The recent finding of cyanide in the blood of animals and man following administration of thiocyanate (4) may indicate the operation of yet other mechanisms. In order to investigate the role of thiocyanate-induced sodium depletion as a possible factor in its hypotensive action, balance studies were undertaken before and during administration of this agent for short periods. For comparison, both normotensive and hypertensive subjects were included.

Methods. Following a control period of 6 or more days on a constant dietary and fluid intake, potassium or sodium† thiocyanate was given by mouth twice daily for 4 days to 16 patients with uncomplicated hypertensive vascular disease and to 26 normotensive subjects. With the exception of 9 members of the hypertensive group who were ambulatory hospital patients, all subjects carried on their customary activities, but took their meals, prepared by the metabolism ward dieticians, under supervision at the hospital throughout the study. Individuals with albuminuria, impaired urinary concentrating ability, diarrhea, fever, complicating respiratory or other infections were excluded from the study. Blood samples were drawn fasting, immediately before the first, and 12 hours following the fourth and eighth doses of thiocyanate. Peak serum thiocyanate concentrations ranged between 2.8 and 6.9 mg per 100 ml. In the normotensive group, the calculated daily sodium intake, constant in each subject, was varied between 75 and 233 mEq in different subjects with a mean of 139 mEq; corresponding values for the hypertensive series were, respectively 18, 227 and 138 mEq. The daily urinary sodium excretion was determined in all subjects, and the output of potassium, chloride and thiocyanate in most. Analysis of serum concentrations of sodium, potassium(5), chloride(6), bicarbonate(7) and thiocyanate(8) was made in a majority of the experiments. Urine volume and body weight were recorded daily, and whole blood cyanide concentrations(9) were measured in two hypertensive subjects.

Results. The average daily urinary excretions of sodium during the last 4 days of the control period and during the 4-day experimental period were compared. An increase over baseline values was observed in most normotensive and all hypertensive subjects, the mean change amounting to 16 and 26 mEq per day, respectively (Tables I and II). Among 26 normotensive subjects, the average daily urinary sodium output during thiocyanate administration exceeded the intake in seven. In contrast, all 16 hypertensive patients went into negative sodium balance during this period.

Small decreases in weight and increases in urine volume were generally observed. Chloride excretion roughly paralleled the sodium output; the urinary potassium remained essentially constant. Alterations in the levels of serum sodium, potassium, chloride bicarbonate and total protein appeared to be neither consistent nor significant. Whole blood cyanide concentrations in two hypertensive patients were 0.05 and 0.1 mg per 100

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† The sodium salt was given to 6 hypertensive patients. Appropriate corrections were made for the additional intake of sodium.

TABLE I.
Sodium Balance in Normotensive Subjects.

Subject	Sodium intake, mEq/24 hr	Avg 24 hr control urine sodium output, mEq/24 hr	Change in sodium output, mEq/24 hr
Group A—Low sodium intake			
1	81	68	—12
2	77	61	+12
3	79	49	+16
4	86	49	+43
5	83	63	— 3
6	83	60	+14
7	88	67	— 2
8	87	52	+21
9	75	52	+15
10	80	80	+ 2
11	83	60	+15
12	80	55	+18
13	90	57	+28
14	96	69	+29
15	79	55	+11
Group B—High sodium intake			
16	214	195	— 3
17	213	192	— 4
18	213	200	0
19	216	211	— 9
20	225	195	+46
21	215	217	+ 8
22	217	197	— 3
23	220	185	+29
24	189	150	+46
25	233	192	+60
26	223	186	+37
Mean values			
Group A	83	60	+14
" B	216	193	+19
" A+B	139	116	+16

ml at serum thiocyanate levels of 4.2 and 5.9 mg per 100 ml, respectively.

Discussion. The observation that thiocyanate administration may be accompanied by a sodium diuresis suggests the possibility that the depressor action of this agent may result, at least in part, from its production of salt depletion. In the hypertensive group, the increase over baseline values of the mean sodium excretion during thiocyanate therapy is highly significant ($P < 0.0001$). It is almost as significant in the normotensive series ($P < 0.0001$), although the difference between the mean changes in the two groups is not ($P = 0.06$). The latter statistics reflect the fact that the average response of the normotensive group is disproportionately increased by the small number of subjects in whom the sodium diuresis greatly exceeds the mean value. The question is therefore raised again whether these "hyperreactors" are in reality in a "pre-hypertensive" stage(10).

The mechanism by which thiocyanate produces a sodium diuresis is not clear, but certain hypotheses can be made. Increases in urine volume during thiocyanate administration to rabbits(11,12) and man (8) were mentioned in early reports. More recent observations have shown a greater increase in urinary chloride concentrations in dogs given thiocyanate than in those receiving other ions in the lyotropic series(13). In view of the conversion of thiocyanate to cyanide *in vivo,* another possible mechanism for the observed diuresis is suggested by the finding that cyanide in small doses is a powerful diuretic in dogs(14). Carbonic anhydrase, an enzyme present in the kidney, is inhibited by thiocyanate as well as cyanide(15); inhibition of this enzyme is accompanied by increased urinary base excretion(16-18). Because of the general belief that thiocyanate is stable, it is of considerable interest that this ion yields measurable amounts of cyanide rapidly *in vitro* in the presence of erythrocytes(19).

The hypotensive action of sodium depletion in hypertensive vascular disease is widely ac-

TABLE II.
Sodium Balance in Hypertensive Subjects.

Subject	Sodium intake, mEq/24 hr	Avg 24 hr control urine sodium output, mEq/24 hr	Change in sodium output, mEq/24 hr
Group A—Low sodium intake			
1	92	84	+13
2	97	100	+15
3	90	82	+12
4	92	75	+20
5	88	83	+14
6	18	16	+22
7	90	84	+ 7
8	87	50	+44
Group B—High sodium intake			
9	182	154	+57
10	207	163	+63
11	132	108	+32
12	219	205	+27
13	225	206	+25
14	225	218	+24
15	227	255	+20
16	132	122	+25
Mean values			
Group A	82	72	+18
" B	194	179	+34
" A+B	138	126	+26

cepted. In the light of the data presented, it is conceivable that the depressor action of thiocyanate may be mediated by this mechanism either directly or as a sequel to its conversion to cyanide. A direct effect of thiocyanate or cyanide on the vascular system, however, cannot be excluded. In either case, it is apparent that the influence of derived cyanide throughout the body as well as upon the kidney must be included in any consideration of the mechanism of action of thiocyanate in hypertensive disease.

Summary. A small but highly significant sodium diuresis accompanied short-term thiocyanate administration to uncomplicated hypertensive subjects on constant dietary regimens. Although slight but insignificant differences were observed between the mean responses of normotensives as compared to hypertensives, it was suggested that the similarity was attributable to the presence of a few hyperreactive and perhaps "pre-hypertensive" individuals in the normotensive series. The conversion of thiocyanate to cyanide *in vivo* was confirmed. Thiocyanate-induced sodium depletion was suggested as a possible factor in the depressor action of this agent, mediated either directly or through derived cyanide.

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Effect of Ether on Certain Coxsackie Viruses.* (19807)

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Soon after the discovery of the Coxsackie viruses by Dalldorf and his associates(1), numerous similarities between these agents and the poliomyelitis viruses were noted(2). It has been emphasized that both are hardy viruses, highly resistant to physical and chemical agents; a notable similarity being their resistance to diethyl ether.

In other studies(3,4) we have been concerned with the *in vitro* effect of diethyl ether on a number of viral agents. Because of the accumulating evidence that the individual members of the Coxsackie family of viruses

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TABLE I. Effect of Ether upon the Ohio-R (Group B, Type 2) Strain of Coxsackie Virus after Contact for 2 Hours at 37°C.

		Final % ether by volume*				
Dilution of virus	Control	5%	10%	20%	30%	50%
10^{-1}			8/8	8/8	16/16	8/8
10^{-2}		8/8†	5/6	6/8	5/16§	6/19
10^{-3}	10/10	8/8	0/6	0/8	0/10	0/16
10^{-4}	9/9	3/8	0/6	0/8	0/6	0/14
10^{-5}	5/11	0/8	0/6		0/5	
10^{-6}	3/14					
10^{-7}	1/13					
LD_{50} titer of virus	$10^{-5.2}$	$10^{-3.4}$	$10^{-2.4}$	$10^{-2.3}$	$10^{-1.7}$	$10^{-1.9}$
Virucidal capacity‡ of ether		1.8	2.8	2.9	3.5	3.3

* Virus-ether mixtures shaken slowly in 37°C water bath for 2 hr before subcut. inj. into 2-day-old mice (inoculum –.05 ml).

† No. of mice which died from C virus infection/No. inoculated (not including those which died from non-specific causes).

§ Three additional mice showed symptoms and recovered.

‡ Value obtained by subtracting log of the LD_{50} of virus in the control from the log of the LD_{50} of virus in the presence of the concentration of ether indicated.

(C viruses) differ among themselves in many respects, it seemed worthwhile to determine the effect of diethyl ether on members of this group to see whether differences might be found among the various serologic types that would allow further classification.

Materials and methods. All C virus strains used in these experiments have been maintained by passage in 2-day-old Swiss albino mice. Three group A strains, type 1 (T.T.), type 2 (Fl.) and type 3 (J.Ol.), were supplied by Dr. Gilbert Dalldorf. The group A, type 4, Dallas-M.B. strain which is serologically similar if not identical to the Texas-1 strain, was isolated in this laboratory(5). The Texas-1 strain and the 3 group B strains, type 1 (Conn.-5), type 2 (Ohio-R), and type 3 (Nancy), were provided by Dr. Joseph L. Melnick. Randomized 2-day-old white Swiss mice (CFl strain) were used throughout these experiments and the Reed-Muench method was used to calculate end-points in the virus titrations. The method of determining the virucidal capacity of ether was similar to that described previously(3). Appropriate serial dilutions of each virus were mixed with varying amounts of diethyl ether† to give total volumes of 2.0 ml per tube. The dilution of the virus and the per cent ether indicated in each experiment represent the final concentration of each in the total volume. A control series of virus dilutions without ether was included for determining the LD_{50} under the conditions of the experiment. The tubes were tightly stoppered immediately after adding the ether and were placed in a 37°C water bath or in the refrigerator together with the controls. At the end of the period of incubation during which time the tubes were inverted from time to time the ether was removed by vacuum distillation or by evaporation prior to subcutaneous inoculation into 2-day-old mice. The inoculum for each animal was 0.05 ml. Ether-treated suspensions were injected first and controls last in order to minimize rather than emphasize any *in vitro* effect of ether. The virucidal capacity of ether was determined by subtracting the log of the LD_{50} of virus in the control from the log of the LD_{50} of virus in the presence of the different concentrations of ether. This difference is actually the log of the amount of virus inactivated.

Results. The group B, type 2, Ohio-R strain was used for the initial experiments because it was found to be more labile than other C viruses(6). Also, this strain seems to differ in other respects from other C viruses (7). Ether was mixed with virus to give final concentrations of 5.0, 10.0, 20.0, 30.0 and 50.0 per cent ether, and final tenfold dilutions of virus ranging from $10^{-1.0}$ to $10^{-7.0}$ and the mixtures were incubated for 2 hours at 37°C. The remaining procedure was the same as described above. Animals were observed frequently during the observation period of 2 weeks for symptoms of C virus infection and death.

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† Diethyl ether, Squibb, USP, known to be free of peroxides and aldehydes, was used exclusively.

It is evident from the results summarized in Table I that ether even in a concentration of 5.0% had some virucidal activity, causing a reduction of 1.8 log units in the LD_{50} of this virus. Higher concentrations of ether ranging from 10.0 to 50.0% were markedly virucidal reducing the LD_{50} titer of the virus by 2.8 to 3.3 log units. It is significant, however, that the highest concentration of virus used (10.0% suspension) was still infective for all mice inoculated after contact with ether for 2 hours at 37°C. This would suggest that the large amount of tissue contained in this suspension may be a limiting factor. This has been noted previously with the St. Louis encephalitis and Eastern equine viruses(3).

There was little evidence of virucidal activity when the ether-virus mixtures were incubated for 2 hours at 4°C despite the greater solubility of ether at the lower temperature. Fifty per cent ether caused a reduction of only 0.6 log unit in the LD_{50} of this virus. However, when this concentration of ether was in contact with the virus for 18 hours at 4°C there was a 1.1 log decrease in the titer of the virus. None of the other C viruses studied, including four group A and two group B serological types, were affected by ether when tested at 37°C or 4°C.

Discussion. It is becoming increasingly apparent that the currently designated Coxsackie family of viruses is a heterogeneous group that requires further classification. The production in suckling mice of a systemic disease predominantly involving skeletal muscle is a feature which all members of this group have in common. Evidence accumulates, however, that individual members of this group differ among themselves in many respects. In addition to differing among themselves serologically(2) and in their pathogenesis in suckling mice(8,9) and in cortisone pretreated adult mice(7), differences in size(10), thermosusceptibility(11,12), effect of pH(11), and even in epidemiologic patterns(13-15) occur among the various types.

The present report calls attention to another feature by which certain C viruses might be differentiated. Of the eight strains of C viruses tested including 5 of group A representing 4 serological types and three of group B, only one strain, the group B, type 2, Ohio-R strain proved to be susceptible to ether. Concentrations of ether ranging from 10.0 to 50.0% were markedly virucidal after contact with the virus for 2 hours at 37°C. The effect of ether was considerably less when the mixtures were incubated at 4°C despite the greater solubility of the anesthetic agent at the lower temperature. This observation would place this strain of C virus, along with the influenza group, arthropod-viruses and lymphogranuloma-psittacosis group in a category of agents which are susceptible to diethyl ether. All other C viruses tested seem to fall in a category of hardy agents, along with the viruses of poliomyelitis, rabies, Col. SK, measles, foot-and-mouth disease, and others, which are notably resistant to ether(4,16).

Variations in the resistance of different strains of poliomyelitis and rabies virus have been noted(17,18). The same may be true of other group B, type 2, C viruses. Furthermore, the resistance of a given strain to the virucidal activity of ether may vary on animal passage(19,20). Rabies *virus fixé,* for example, is usually more susceptible than street virus to *in vitro* destruction by this agent. Whether or not early passages of the Ohio-R strain of C virus were less susceptible to the effects of ether could not be determined since this material was not available for study.

The Ohio-R strain was isolated originally from specimens which had been treated with penicillin-streptomycin mixtures to remove concomitant organisms. Many investigators, however, use ethyl ether and occasionally submit specimens to triple ether treatment to remove contaminating microorganisms. The type 2, group B Coxsackie virus has been isolated less frequently than other serologic types. Since clinical specimens presumably contain only small amounts of virus it is likely that ether treatment may have influenced the frequency with which this serologic type of C virus has been encountered even though this agent is only weakly sensitive to ether at 4°C.

Summary. Of 8 strains of Coxsackie viruses tested including 5 of group A representing four serological types and 3 of group B, only one strain, the group B, type 2, Ohio-R strain proved to be susceptible to diethyl ether. In addition to serving as a means of differentiating the Ohio-R strain from other serological types of C viruses, the use of ether may be a limiting factor in the isolation of this agent.

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Interference Between Japanese B Encephalitis Virus and Western Equine Encephalomyelitis Virus in the Rat. (19808)

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It has recently been reported(1,2) that 3- to 4-week-old rats, which are highly susceptible to intranasally instilled Western equine encephalomyelitis (W.E.E.) virus, are much less susceptible if St. Louis encephalitis (S.L.E.) virus is instilled into their noses 72 hours prior to the inoculation of W.E.E. virus. The purpose of the studies reported here was to determine whether Japanese B encephalitis virus which, like S.L.E. virus, induces a fatal infection in 7-day-old but not in 21-day-old rats(3) would "interfere" with W.E.E. virus following intranasal inoculation of both viruses.

Materials and methods. Viruses and animals. The Japanese B encephalitis virus used was the Nakayama strain which had been carried through an unknown number of intracerebral passages in mice. The strain of W.E.E. virus used has already been described (1,2). The virus suspensions were prepared from virus-infected brains of 2- to 3-week-old Swiss mice. The infected brains were placed in a Waring blendor (semi-micro size jar) together with sufficient cold, inactivated, undiluted rabbit serum to make a 10% suspension of the infected brains. The blendor was

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TABLE I. Interference Between Japanese B Encephalitis Virus and Equine Encephalomyelitis Virus Following Intranasal Inoculation of Both Viruses.

No. of rats	Material instilled into nose	Interval (days) between W.E.E. and death																Results	% mortality
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
21	Jap. B × 3[*] W.E.E.[†]	0[¶]	0	0	2	0	0	0	0	1	0	0	0	2	1	1		7/21[**]	33
20[‡]	Jap. B × 1[§]	0	0	0	1	1	0	1	0	3	1	0	0	1	0	0	2	10/20	50
20	N.M.B. × 3[‖]	0	0	16	3	1												20/20	100

[*] Jap. B × 3. Three daily inoculations of Japanese B encephalitis virus prior to inoculation of equine encephalomyelitis virus.

[†] W.E.E. Western equine encephalomyelitis virus.

[‡] One animal in this group became paralyzed but survived.

[§] Jap. B × 1. A single inoculation of Japanese B encephalitis virus prior to inoculation of equine encephalomyelitis virus.

[‖] N.M.B. × 3. Three daily inoculations of normal mouse brain prior to inoculation of equine encephalomyelitis virus.

[¶] Zero indicates no deaths on that day. Other number indicates number of deaths on that day.

[**] Denominator indicates No. of animals tested. Numerator indicates No. of deaths.

run for 90 seconds and the resulting suspension then centrifuged at about 2000 RPM for 5 minutes to sediment gross particles. The supernatant fluid was removed, distributed in 2 ml amounts in screw-capped vials, "quick frozen" in a mixture of dry-ice and alcohol and then stored in a CO$_2$ cabinet until needed. A single suspension of each of the 2 viruses was used. The mouse intracerebral LD/50 titer(4) of the suspension of Japanese B encephalitis virus, after storage in the CO$_2$ cabinet for 7 days, was 1 x 10$^{-8.4}$. The W.E.E. virus, following 9 days storage in the CO$_2$ cabinet, titered 1 x 10$^{-8.8}$. The 10% normal mouse brain (N.M.B.) suspension was prepared by grinding brains from normal 2- to 3-week-old mice in a mortar with sand and sufficient cold, inactivated, undiluted rabbit serum to make a 10% suspension. It was then treated as described for the preparation of the virus suspensions. Male Sprague-Dawley rats which were shipped when 21 days of age were used. They weighed 38 to 54 g, with an average weight of 45 g at the time they were used. All inoculations were made while the rats were under light ether anesthesia.

Experimental. The 61 rats used in the experiments were divided into 3 groups and inoculated intranasally with a 10% suspension of the material being tested (Table I). One group, consisting of 20 rats, received 0.05 ml of the N.M.B. suspension on each of 3 consecutive days. These animals then received 0.05 ml of the suspension of W.E.E. virus 72 hours after the first inoculation of N.M.B. A second group of 20 animals received a single inoculation of 0.05 ml of the suspension of Japanese B encephalitis virus followed 72 hours later by a single inoculation of 0.05 ml of W.E.E. virus. The third group consisting of 21 rats, received 0.05 ml of Japanese B encephalitis virus on each of 3 consecutive days. These animals were then inoculated with 0.05 ml of W.E.E. virus 72 hours after they had received the first inoculation of Japanese B encephalitis virus. The schedule of inoculations was arranged so that all 3 groups were inoculated with the W.E.E. virus on the same day. The rats were observed for 21 days. The results are presented in Table I and show that the group of 20 rats which received 3 inoculations of N.M.B. before the W.E.E. virus was inoculated were uniformly susceptible to the virus since all of the animals succumbed. The results were different, however, in the 2 groups of animals which had had Japanese B encephalitis virus instilled into their noses before the W.E.E. virus was inoculated. Of the 20 rats which received a single inoculation of Japanese B encephalitis virus followed by W.E.E. virus only 10 of the 20 animals developed a fatal infection. However, one of the 10 animals which survived in this group did not completely escape infection with W.E.E. virus. On the 13th day following inoculation with W.E.E. virus this animal developed flaccid paralysis of both posterior extremities as well as the right front leg. Three days later the animal regained the use of the posterior extremities but irreparable damage had occurred because the animal never recovered the use of

TABLE II. Susceptibility of Rats to a Second Intranasal Inoculation of Equine Encephalomyelitis Virus.

No. of rats	Material instilled into nose	Interval (days) between 2nd inoc. of W.E.E. and death 1	2	3	4	5	Results	% mortality
14	Jap. B × 3 W.E.E. × 2*	0	0	9	3	1	13/14	93
9	Jap. B × 1 W.E.E. × 2	0	0	5	2	1	8/9	89

* Rats reinoculated intranasally with W.E.E. virus 23 days after having previously received W.E.E. by the same route.

the right front leg since muscular atrophy, giving rise to contractures, occurred. It should be noted that this is the first instance in which we have observed a non-fatal infection in the rat caused by W.E.E. virus. In the group of rats which received 3 inoculations of Japanese B encephalitis virus before the W.E.E. virus was inoculated a high degree of resistance was noted since only 7 of the 21 animals tested were found to be susceptible to the W.E.E. virus.

Twenty-three days after W.E.E. virus was instilled into the noses of the above rats, certain of those which survived were reinoculated intranasally with W.E.E. virus to determine whether the original inoculation of W.E.E. virus had rendered them resistant to a second inoculation by the same route. The results appear in Table II and show that the animals which had received 3 inoculations of Japanese B encephalitis virus as well as those which received only one before the original inoculation of W.E.E. virus were highly susceptible to a second intranasal inoculation of W.E.E. virus since only one animal in each group survived.

Discussion. In previous communications (1,2) it was reported that the susceptibility of rats to intranasally instilled W.E.E. virus was altered if S.L.E. virus were inoculated into the noses of the animals before they received the W.E.E. virus. If one inoculation of S.L.E. virus preceded the W.E.E. virus 50 to 65% of the animals survived whereas 95% of the rats were found to be resistant if 3 inoculations of S.L.E. virus preceded the W.E.E. virus. Since S.L.E. virus invades the central nervous system of rats of this age by passing along the olfactory nerve to establish an inapparent infection(5,6) it was postulated that S.L.E. virus, having passed over the olfactory tract and being present in the brain, prevented the subsequent passage of W.E.E. virus over this pathway. Evidence has been obtained which suggests that Japanese B encephalitis virus invades the central nervous system of 3- to 4-week-old rats by the olfactory route following nasal instillation of the virus since the virus was recovered from the anterior rhinencephalon as early as 24 hours after inoculation when it was not recovered from the "remainder of brain" or from the spinal cord(7). It is possible then that the mechanism by which both S.L.E. and Japanese B encephalitis viruses protect a high percentage of rats against intranasally inoculated W.E.E. virus is essentially the same since it would appear that the passage of either of these 2 viruses along the olfactory pathway interferes with the subsequent passage of W.E.E. virus along this route. The observation that the Japanese B virus-treated rats which survived the original inoculation of W.E.E. virus were highly susceptible to W.E.E. virus instilled intranasally 23 days later indicates that the mechanism by which Japanese B encephalitis virus interferes with W.E.E. virus functions in such a manner that the W.E.E. virus is unable to immunize the animals against a second inoculation of W.E.E. virus.

Summary. If Japanese B encephalitis virus is instilled into the noses of 3- to 4-week-old rats 72 hours prior to the inoculation of Western equine encephalomyelitis virus by the same route a high percentage of the animals resist infection with the latter virus. If one inoculation of Japanese B virus preceded the Western equine virus 50% of the rats survived whereas 67% of the animals survived when 3 inoculations of Japanese B virus preceded the equine encephalomyelitis virus. Rats which survived when equine encephalomyelitis virus was instilled into their noses 72 hours following inoculation of Japanese B encephalitis virus by the same route were found to be susceptible to a second intranasal inoculation of equine encephalomyelitis virus.

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The Size of Serum Hepatitis Virus.[*] (19809)

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The agent of serum hepatitis (SH) is known to pass through various "bacteria-retaining" filters (Seitz EK(1,2) and Berkefeld N(3)), but the size of the virus, like that of infectious hepatitis (IH), has so far remained undetermined. The present report is concerned with experiments designed to determine the size range for SH virus by means of ultrafiltration of known icterogenic serum through a series of gradocol membranes[†] of various average pore diameters (A.P.D.)(4).

Materials and methods. Acute phase serum (strain J. W.) was obtained from an American soldier in Germany on the second day of jaundice (30 September 1949). It was kept in a CO_2 dry ice box for 42 days and then at –20°C until the filtrations were carried out in July and November of 1951.[‡] A portion of this same lot of serum had previously (1950) produced clinical and laboratory evidence of hepatitis in 3 volunteers, each of whom received 0.5 cc subcutaneously and 0.1 cc intracutaneously(5). The incubation period at that time ranged from 53 to 87 days. In the first series of filtrations whole undiluted serum was used. After clarification through a Seitz EK pad a portion of the serum was passed through a 300 $m\mu$, A.P.D. gradocol membrane under 12 lb positive pressure (nitrogen gas) at 38-39°F. This was then used as a "master" filtrate, portions of which were filtered through membranes of 200 $m\mu$, and 94 $m\mu$ A.P.D. under similar conditions. In the second series of filtrations 10% serum in normal saline was used, the procedure being the same as above except for the addition of a 52 $m\mu$ membrane. This dilution factor was tried in order to facilitate filtration which is a most time-consuming procedure when whole undiluted serum is used. All filtrates were kept frozen until the time of inoculation. Twenty-nine volunteers, quartered in the same institution,[§] participated in the present study. All were healthy males (aged 21 to 30) who gave no history of previous jaundice of any type. All had normal liver function tests (*i.e.*, direct and total serum bilirubin, cephalin fluocculation, thymol turbidity and flocculation, and alkaline phosphatase) at the time of inoculation.

Exp. I. Seventeen volunteers were divided into 4 groups, each receiving a single filtrate

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[†] Obtained from the Inoculation Department, St. Mary's Hospital, London.

[‡] Filtrations were carried out with the aid of Dr. Joel Warren in the Virus and Rickettsial Disease Laboratory, Army Medical Service Graduate School, Washington, D.C.

[§] Federal Correctional Institution, Danbury, Conn. Acknowledgment is made to Mr. Allen Shank and Mr. L. C. Schilder, Wardens, and Dr. Leon Witkin, Chief Medical Officer, for their permission to allow these men to volunteer and for their generous assistance and cooperation during the observation period.

TABLE I.
Summary of Results with Various Filtrates.

		Whole serum	10% serum
Unfiltered		+ + +*	Not used
Filtrate	Seitz EK	+ + — —	” ”
	300 mμ	+ + — —	” ”
	200	± — — — —	+ — — —
	94	± — — —	+ — — —
	52	Not used	+ — — —

Each sign (+, ± or —) indicates one volunteer inoculated.

+ = definite laboratory and/or clinical evidence of hepatitis.

— = no evidence of hepatitis.

± = borderline elevations of serum bilirubin on one occasion only.

* Three tests representing an earlier exp.(5).

of whole serum (Seitz and membrane filters of: 300 mμ, 200 mμ, or 94 mμ). The inoculum consisted of 0.4 cc subcutaneously and 0.1 cc intracutaneously. *Exp. II.* Twelve volunteers were divided into 3 groups, each receiving a single filtrate of 10% serum in normal saline (200 mμ, 94 mμ, or 52 mμ). The total inoculum used was 5 cc given in multiple subcutaneous injections. During the post-inoculation period in both experiments all volunteers were interviewed and examined at approximately weekly intervals. Liver function tests were repeated 3 and 6 weeks after inoculation and at one to 2 week intervals thereafter until the termination of the experiment at the end of 4 months. Individually numbered syringes and needles were used in order to avoid any possibility of cross inoculation. The volunteers were not isolated except during the period of acute illness. No cases of hepatitis had been observed in the institution for one year prior to the present study and none was observed outside the experimental groups during the period of observation.

Results. The rates of occurrence of hepatitis among all of the inoculated volunteers are listed in Table I.

In the first experiment 2 of the 4 individuals receiving the Seitz filtrate and 2 of the 4 receiving the 300 mμ filtrate developed definite clinical and/or laboratory evidence of hepatitis. The degree of illness varied from extremely mild to moderately severe, with peak serum bilirubin levels ranging from 2.06 to 7.92 mg %. The average incubation period was 76.5 days, ranging from 73 to 87 days, and the average duration of illness was approximately 35 days. One member of each of the other groups (200 mμ and 94 mμ) showed borderline elevations of serum bilirubin levels at the same time that the above cases first became apparent. These "borderline" values were 2 to 3 times the levels previously observed in the same individuals, but they fell rapidly to normal and there were no other confirmatory laboratory and no clinical findings.

In the second experiment definite clinical and laboratory evidence of hepatitis developed in one member of each of the 3 groups (200 mμ, 94 mμ, and 52 mμ) of 4 inoculated volunteers. Each of these men became markedly icteric (peak serum bilirubin levels from 13.6 to 23.1 mg %) and the clinical course was much more severe than in Exp. I. Because of the marked nausea and vomiting and the difficulties involved in providing adequate parenteral nutrition, these men were all treated with cortisone (100 mg intramuscularly for 5 days, followed by the oral preparation in gradually decreasing dosage during the next 20 days). Coincident with this therapy, there was a prompt and dramatic subsidence of symptoms within the first 24 hours. Serum bilirubin levels decreased fairly rapidly during the first few days and continued a steady downward trend in 2 of the patients as the cortisone dosage was reduced. In the third patient, however, a sudden relapse occurred with the decrease in dosage, followed by prompt improvement when the dosage was increased. No relapse was noted during a period of 2 months after cortisone was discontinued in these 3 patients. The incubation period in this group ranged from 62 to 70 days, averaging 65 days. The duration of illness in each case was approximately 50 days.

A summary of the clinical and laboratory findings of both experiments, together with the results previously obtained by Evans(5) using the same (J.W.) serum unfiltered, is given in Table II.

Discussion. In the first experiment the results were equivocal with a questionable endpoint reached with the 300 mμ filtrate. It was felt, however, that whole serum might have produced sufficient plugging of the smal-

TABLE II.
Clinical and Laboratory Findings in 32 Volunteers Inoculated with SH Virus (J.W. Strain).

	Inoculum	No. with hepatitis /No. in group	Men who devel. abnormal liver function tests	Day of onset of symptoms	Day of onset of icterus	Liver exam. Enlargement	Liver exam. Tenderness	Serum bilirubin* (highest level) Direct, mg %	Serum bilirubin* (highest level) Total, mg %	Avg peak serum bilirubin for group
Evans(5)	Unfiltered whole J.W. serum (.5 cc subcut. .1 cc intracut.)	3/3	FT	53	53	no	yes	1.15	2.96	6.96
			BS	65	68	no	yes	4.90	9.96	
			CH	75	87	yes	yes	3.29	7.57	
Exp. I	Filtrates of whole J.W. serum (.4 cc subcut. .1 cc intracut.)									
	Seitz EK	2/4	JT	0	0	?	?	.89	2.06	5.12
			DS	75	80	yes	yes	3.53	5.98	
	300 mμ	2/4	WC	85	93	yes	yes	4.41	7.92	
			HL	82	95	yes	yes	2.53	4.52	
	200 mμ	0/5	TK†	0	0	no	no	.15	1.08	
	94 mμ	0/4	JB†	0	0	no	no	.24	1.38	
Exp. II	Filtrates of 10% J.W. serum (5 cc subcut.)									
	200 mμ	1/4	PM	60	62	yes	yes	11.60	23.1	17.27
	94 mμ	1/4	WM	75	77	yes	yes	7.42	15.1	
	52 mμ	1/4	RN	68	72	yes	yes	7.16	13.6	

* Upper limits of normal in this laboratory: .20 mg % direct; 1.30 mg % total.

† These results are included only because a "suggestive" rise in serum bilirubin levels occurred at the same time that abnormal levels were noted in other volunteers in the same experiment.

ler A.P.D. membranes to prevent passage of an infective dose of virus. That this was probably true was borne out by the second experiment using 10% serum filtrates which yielded evidence that the virus passed through the smallest (52 mμ) A.P.D. membrane used.[‖] According to Elford's formula(6) the size of the virus would be 26 mμ or less. Since no end-point was reached the size range cannot be further limited.

It has been shown that the pores of collodion membranes of the type used are not uniform in size or configuration, that there is a wide variation in the pores of a single membrane(7), and that there may be variations in results obtained using membranes of the same production lot(8). Therefore, the results of a single experiment are open to considerable question and the value of 26 mμ or less is given with these facts in mind.

Another aspect of the results of these experiments should be noted, although it is again realized that the numbers involved in the tests are small and of questionable significance. When 10% serum filtrates were used instead of whole serum, the three illnesses produced were apparently more severe, of longer duration, and the incubation periods were relatively shorter. Since there is no way of knowing the variations in susceptibility of the indi-

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[‖] Recent experiments(9) in this laboratory using filtrates of Coxsackie virus (size 15 to 23 mμ, titer $10^{-7.5}$) suspended in varying concentrations of horse serum indicate that extraneous protein interferes with passage of the virus, but that if the virus concentration is sufficiently high, there will be no effect on the filtration end point. Whole serum could not be filtered through the smaller A.P.D. membranes satisfactorily. The following table summarizes the results:

Filtrate, mμ	Negative logarithm of virus in Whole horse serum	Negative logarithm of virus in 10% horse serum	Negative logarithm of virus in 1% horse serum
140	5.1	>6.8	>6.8
87	5.5	6.5	>6.8
53	*	2.5	6
41	*	0†	0†

* Serum not filterable.

† 0 indicates max conc. of virus tested (.5% suspension of infected tissue) failed to infect any of 24 infant mice.

viduals involved, no definite conclusions can be drawn from these observations and any explanation falls into the realm of speculation. It is suggestive, however, of a change in the infectivity of the virus in the process of dilution, perhaps concerned with a virus-antibody relationship.

Cortisone therapy appeared to have a definite and immediate beneficial effect in the three patients who were treated, but any conclusions concerning its value from this study would again be questionable since there was no adequate control group and the number of cases was small.

Summary. 1. Known icterogenic (SH) serum (J.W. strain), undiluted and diluted 10% in normal saline, was filtered through gradocol membranes of various average pore diameters down to 52 mμ, and tested in human volunteers. 2. Evidence has been presented that the strain of serum hepatitis virus used is small enough to pass through a 52 mμ A.P.D. membrane, thus indicating a size of 26 mμ or less. The limitations of the application of this method for determining the size of this virus are mentioned.

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In Vitro Conversion of Thiocyanate to Cyanide in the Presence of Erythrocytes.* (19810)

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The *in vivo* conversion of thiocyanate to cyanide has been reported recently by Goldstein and Rieders(1) in contrast to previous studies in which this reaction could not be demonstrated(2,3). Because thiocyanate has been widely used in the management of patients with hypertensive vascular disease, further study of this problem was undertaken in the course of investigation of thiocyanate-induced diuresis in man(4).

Methods. Cyanide was determined by the method of Gettler and Goldbaum(5). The material to be tested is acidified, heated to 90°C and aerated; the eluted hydrogen cyanide is carried to filter paper impregnated with ferrous sulfate, producing the Prussian blue reaction. The method is reported to be highly specific and sensitive to 0.0001 mg. For the purposes of the present study no effort was made to obtain sensitivity greater than 0.0005 mg. Standards were set up in whole blood and serum containing 0.0005, 0.001, 0.002, 0.003, 0.004, and 0.005 mg of cyanide;† the colored papers were preserved for comparison with the unknowns. The specificity of the method appeared to be adequate as judged by a limited number of observations. Repeated analyses of water, serum, plasma, and boiled whole blood to which varying amounts of thiocyanate had been added yielded no detectable cyanide. The development of the characteristic odor and the conversion of methemoglobin to cyanmethemoglobin (by spectrophotometric analysis) as well as the starch-iodine and copper-benzidine acetate tests(5a) confirmed the presence of cyanide in instances where the test was positive. Thiocyanate was determined by the method of Barker(6) adapted for use with the Coleman spectrophotometer. Methemoglobin was estimated by the method described by Evelyn and Malloy(7). Known amounts of potassium thiocyanate were introduced into test tubes containing 2 ml of heparinized whole blood or other fluid, or equivalent weights of tissue, at room temperature. The cyanide determination was completed in most instances within 10 minutes of the addition of thiocyanate. Measurements were also made on the fasting whole blood and serum of 2 hypertensive patients before and during administration of therapeutic doses of potassium thiocyanate. The analyses were repeated in one of these patients on another occasion at which time thiocyanate was given during the production of methemoglobinemia by oral doses of p-aminopropiophenone(8). Whole blood cyanide was estimated in 2 hypertensive subjects who had been given sodium nitroprusside, a depressor agent reported to give rise to thiocyanate in man(9).

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† The terms "cyanide" and "thiocyanate" refer to the anion only.

Results. The introduction of potassium thiocyanate into heparinized human or rabbit whole blood *in vitro* was followed within 10 minutes in each 50 analyses by the appearance of measurable amounts of cyanide in the red blood cells. No detectable cyanide was observed in the plasma in these instances. The affinity of erythrocytes for this compound was further indicated by measurements on whole blood after the addition of cyanide; in these cases, cyanide first appeared in the plasma after the concentration in whole blood had been increased to 0.2 mg per 100 ml.

The conversion of thiocyanate to cyanide was also observed in hemolysed blood, but not in previously boiled whole blood. Cyanide was not detectable following the addition of thiocyanate to plasma, serum, water, rabbit liver homogenate or minced rabbit kidney. Nor were measurable amounts of cyanide obtained from solutions of thiocyanate in whole blood containing as little as 2.1% methemoglobin, presumably owing to the formation of stable cyanmethemoglobin (Table I).

TABLE I. Cyanide Concentrations Following the Introduction of Potassium Thiocyanate *In Vitro.*

Medium		Initial thiocyanate, mg/100 ml	Cyanide yield, mg/100 ml
Human whole blood		0	0
		5	.2
		20	.2
		100	.4
Human whole blood	boiled	50	0
	hemolysed	280*	.56*
	+ 2.1% methemoglobin	22	0
Human serum		20	0
		250	0
Water		20	0
		250	0
Rabbit	kidney, minced	110†	0
	liver, homogenized	125	0

* Corrected for dilution factor of 2.8.
† mg per 100 g.

TABLE II. Serial Serum Thiocyanate and Whole Blood Cyanide Levels in Hypertensive Patients Receiving Therapeutic Doses of Potassium Thiocyanate by Mouth.*

Patient	Day	Serum thiocyanate, mg/100 ml	Methemoglobin, %	Whole blood cyanide, mg/100 ml	Serum cyanide
1	1	0		0	0
	3	4		.05	0
	5	4.2		.05	0
2	1	.2		0	0
	3	3.6		.05	
	5	6.1		.1	
During induced methemoglobinemia					
2	1	.1	1.9	0	
	3	3.2	6.9	0	
	5	5.4	2.1	0	

* Determinations were made on fasting blood drawn just prior to the beginning of therapy, after two and after four days of thiocyanate administration.

Serial determinations of serum thiocyanate and whole blood cyanide during thiocyanate therapy (Table II) indicated that cyanide was present when thiocyanate concentrations were approximately half the recommended therapeutic level(6). As anticipated, no cyanide was found in the blood of the patient with methemoglobinemia; it was observed, however, in the blood of both patients given nitroprusside coincident with the appearance in the serum of thiocyanate derived from the nitroprusside.

Discussion. These data confirm the observation of Goldstein and Rieders that thiocyanate is partly converted to cyanide, and indicate further that the reaction is rapid and occurs *in vitro* in the presence of a constituent of the red blood cell. The integrity of the erythrocyte is not essential to the conversion, and the system is heat labile. The evidence thus is compatible with the concept that the reaction is enzymatic, and that the responsible enzyme occurs in the red blood cell. The failure of the conversion to take place in the presence of rabbit liver or kidney is presumptive evidence that these tissues do not contain the enzyme. Lang has demonstrated the presence in liver homogenates of "rhodanase," an enzyme capable of irreversibly synthesizing thiocyanate from cyanide in the presence of colloidal sulfur or thiosulfate. No "rhodanase" was found in muscle or blood(10). It is conceivable, but unlikely in the absence of added sulfur, that the small quantities of cyanide formed from thiocyanate in liver preparations are rapidly reconverted by the "rhodanase" system, leaving none to be detected by the procedure used.

Although well below lethal concentrations, the levels of cyanide observed in the present study are sufficiently high to produce functional changes at least in the kidney. A dose of 0.03 mg of cyanide (0.05 mg of sodium cyanide) per 100 g of body weight has been reported to produce a sodium diuresis in dogs made resistant to mercurials(11). Estimates of the minimal lethal dose of cyanide range between 0.3 mg per 100 g of body weight in dogs(12) to 0.05 mg per 100 g in man(13). A blood level as low as 0.34 mg per 100 ml was observed by the latter authors in a fatal human case of cyanide poisoning. Blood levels in cases of non-fatal poisoning were not found in published reports, but it is probable that these would lie within the range of the concentrations observed following conversion from thiocyanate. Certainly the signs of toxicity from cyanide and thiocyanate are similar in animals and man, as previously noted(1).

The absence of detectable amounts of cyanide from the plasma of blood containing small amounts does not preclude the transfer of red cell cyanide to take part in reactions elsewhere. It is well known that cyanide combines with methemoglobin to form cyanmethemoglobin, a derivative with a characteristic absorption spectrum. This compound was formed within 10 to 15 minutes of the addition to methemoglobin of erythrocytes containing cyanide derived from thiocyanate.

Cyanide and thiocyanate have certain properties in common including their pattern of toxicity in animals and man(1), the production of sodium diuresis(4,11), and inhibition of carbonic anhydrase(14). These similarities support the suggestion(1) that cyanide may account in part for some effects, *in vitro* as well as *in vivo,* formerly ascribed to thiocyanate.

Summary. The conversion of thiocyanate to cyanide was confirmed, and evidence was presented indicating that this reaction occurs rapidly *in vitro* in the presence of a red blood cell constituent, possibly an enzyme. Physiologically significant concentrations of cyanide were observed in the blood of patients with serum thiocyanate values even below therapeutic levels. The evidence was felt to support the suggestion that the biological effects generally ascribed to thiocyanate may be caused in part by cyanide.

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Determination of Oxidative Enzymes and their Inhibitors by a Modified Chromatographic Method. (19811)

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Present-day methods of detecting the action of melanin-forming enzymes and of evaluating the effect of enzyme inhibitors in these systems usually involve the incubation of enzyme-substrate mixtures and subsequent colorimetric determination of the melanin formed. Thus the activity of an anti-metabolite may be expressed as the decrease in melanin formation compared with that exhibited by control enzyme solutions. The sensitivity of this method depends not only upon enzyme concentration but also upon the diluent, the surface-volume ratio, temperature, etc. By this procedure, the anti-melanotic action of thiourea has been estimated at a dilution of approximately 1 x 10^{-4}(1-4). In view of the limitations of this test, a more sensitive method for the study of oxidase and anti-

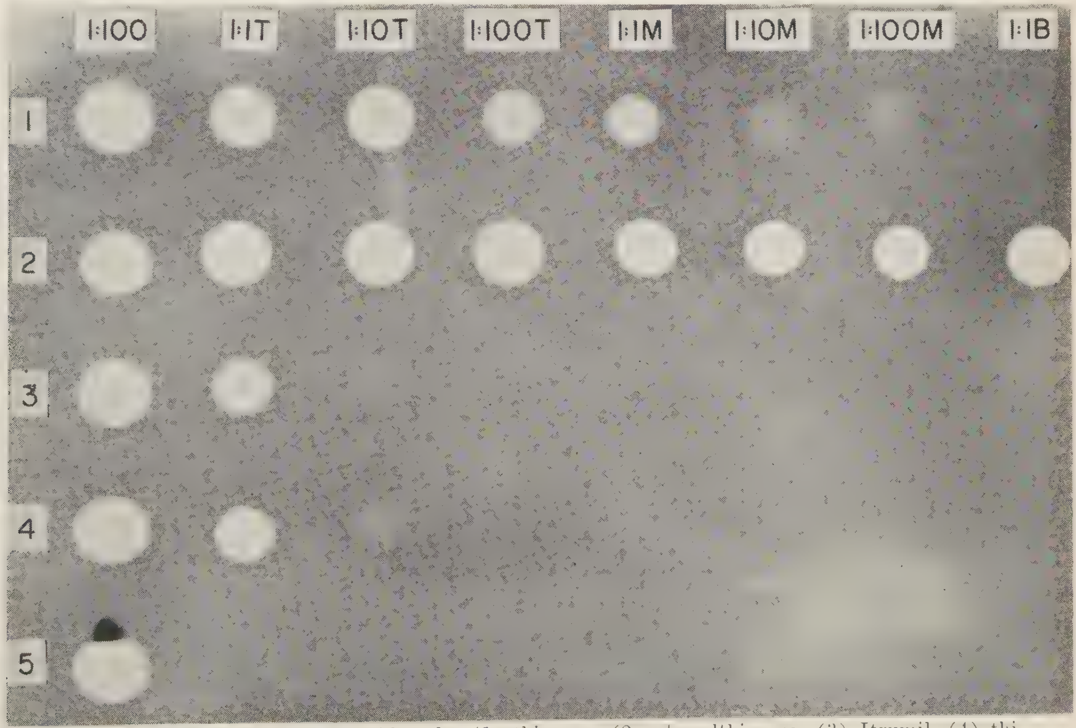

FIG. 1. Inhibition of phenol oxidase by (1) thiourea, (2) phenylthiourea, (3) Itrumil, (4) thiouracil, (5) propylthiouracil.

oxidase activities was developed and is described in this paper.

Experimental. 1. *Detection of enzyme activity.* Tyrosinase (potato phenol oxidase) obtained from fresh potatoes by homogenization under nitrogen was added to an appropriate substrate such as tyrosine, dihydroxyphenyl L-alanine (dopa), or epinephrine and quickly sprayed, under nitrogen pressure, on a large sheet of No. 1 Whatman filter paper. The wet paper was then suspended in a humid chromatographic chamber and observed for color changes. Hallachrome and subsequently melanin developed on the filter paper by oxidation. The shade and rate of filter paper coloration varied with the type of substrate employed. With dopa or epinephrine as melanogens, visible oxidation started immediately. The red color of the ortho-quinone derivatives described by Raper(5) rapidly developed and subsequently changed into the brown and black color of pro-melanin and melanin. With tyrosine as substrate, the oxidation was much slower. At least 30 minutes were required to produce a visible reaction and the color sequence from red-brown to black.

2. *Evaluation of anti-oxidants.* In the manner of a paper chromatogram, drops of serial dilutions of the anti-melanogenic substances to be tested were deposited on filter paper and allowed to dry. The enzyme-substrate solution was then sprayed as described above. After an interval, depending on the substrate used, anti-oxidase activity was manifested as round white spots on a brown or a black background, and the anti-melanotic potency indicated by the highest dilution suppressing complete coloration. Repeated experiments showed that the end points are reproducible and permitted quantitative determination of oxidative enzyme inhibition. The suppression of phenol oxidase by several compounds in shown in Fig. 1.

Results. Employing this method, the extent of anti-oxidase activity of the following compounds was observed:

Phenylthiourea	1×10^{-10}
Thiourea	1×10^{-9}
Thiouracil	1×10^{-4}
Propylthiouracil	1×10^{-3}
5-iodo-2-thiouracil (Itrumil)	1×10^{-4}
Sulfathiazole	1×10^{-3}
Sulfanilamide	1×10^{-3}
p-Aminobenzoic acid (PABA)	1×10^{-3}
p-Aminosalicylic acid (PAS)	1×10^{-4}
p-Acetylaminobenzaldehyde thiosemicarbazone (Tibione)	1×10^{-5}

Phenylthiourea, by this method, exhibited inhibition of phenol oxidase at a dilution greater than 1 x 10^{-10}. Below 1 x 10^{-8} concentrations and with tyrosine as substrate the anti-oxidase effect lasted from ½ to 2 hours and subsequently disappeared. In higher concentrations, *i.e.*, greater than 1 x 10^{-7}, the anti-oxidase activity was complete and permanent.

PABA, PAS and sulfonamides such as sulfanilamide and sulfathiazole suppressed melanin formation in concentrations of 7 x 10^{-2} and 1 x 10^{-4}. These substances caused bright yellow and yellow-brown colorations similar to the yellow pigments formed by Mycobacteria in the presence of PABA and PAS(6,7), and from sulfathiazole from Staphylococci(8). Isonicotinhydrazide (Rimifon) also acted as a potent inhibitor and brought about an orange coloration. The yellow and orange compounds are most likely oxidation-products of these various aromatic amines and formed in a sequence of reactions: tyrosine is transformed to the quinoid hallochrome which in turn reacts with PABA, sulfonamides, and Rimifon.

Summary. A simple method for the detection of potato phenol oxidase and the quantitative determination of inhibitors to this and similar systems yielding color variations is described. Melanin is developed on filter paper sheets and anti-melanotic substances tested by a chromatograph, spray-like procedure.

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Antigenic Study of Certain Slow Lactose Fermenting *Aerobacter cloacae* Cultures. (19812)

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Stuart, Wheeler, Rustigian and Zimmerman (1) reported an extensive biochemical and serologic study of paracolon bacilli in which they examined, among others, 140 strains having the general characteristics of Aerobacter except that they failed to ferment lactose or fermented this sugar only after several days. They called these strains paracolon Aerobacter and classified them into two divisions on the basis of their IMViC reactions. Each division was divided further into types on the basis of other biochemical reactions. In their first division, consisting of 48 cultures, they established 3 biochemical types and in their second division, containing 92 strains, 7 types were recognized. They designated these biochemical types simply by the numbers of typical strains in each type. The authors then prepared serums with a single representative strain from each of the biotypes except two, omitting one in each division, and performed agglutination tests in an effort to reveal the serologic homo- or heterogeneity of the biotypes. On the basis of these serologic studies they reported little serologic homogeneity in the biotypes of their first division, but implied that a high percentage of strains constituting each of the types in their second division were "antigenically identical or closely related." Since, in the preparation of their serums, Stuart and his co-workers used live cultures as antigens and tested the strains in each biotype only with the serum prepared with the type strain it is obvious that their study cannot be accepted as a basis for determining complete antigenic relationships. The methods employed by them are not given in sufficient detail to determine whether agglutination was of the O or the H type. It seems most probable that some titers were due chiefly to H, others to O antibodies. It appears from the findings of Stuart that considerable medical importance can be attached to certain biochemical types which have been classified in the second division. Type 32011 was incriminated as an inciting factor in gastroenteritis and type 37511 was on more than one occasion mistaken for a Salmonella. Both types were the most important members of the second division when considered on the basis of frequency of isolation from patients.

The cultural characteristics of the paracolon aerobacters are similar in many respects to normal *A. cloacae* cultures. In addition a number of investigations in the past have suggested that *A. cloacae*-like bacteria may be associated with a variety of human diseases (2-7). It was decided, therefore, that a more specific study of the antigens of the Stuart cultures would be desirable. Because of their similarity to normal *A. cloacae* cultures some strains of this species were introduced into the study for comparison purposes.

Methods. The following 32011 cultures were obtained from Dr. C. A. Stuart: SRC, 63511, 70811, 68411, C2, C3, C5, C6, and C7. Obtained from Stuart's collection also were cultures 32821, 35611, 37211, 37511, and 37711 which were described as differing biochemically from type 32011 but related antigenically. The following *A. cloacae* cultures were obtained from the American Type Culture Collection. ATC No. 222 (E. O. Jordan) and ATC No. 962 (M. Levine). Dr. W. H. Ewing supplied 2 paracolon aerobacter cultures, No. 2556 and 979 which had been received by the enteric laboratory, Communicable Disease Center, Atlanta, Ga., for identification as possible pathogens. All of the above cultures were observed for purity and evidence of serologic roughness. A number of other cultures obtained for use in this study could not be included because of an inability to obtain S forms. When tested biochemically, the Stuart cultures conformed to their original description. The Ewing cultures fermented lactose within 5 days and the *A. cloacae* cultures obtained from the American Type Culture Collection showed lactose fermentation in 48 hours. All of the cultures fermented glycerol without gas formation within 5 days and all failed to ferment inositol within a 10-day observation period(8). In the serologic study of this group of cultures the well-known methods for the identification of O, H and K type antigens were used and attention was given to the possible existence in the cultures of the alpha type antigen of Stamp and Stone(9). *Thermostable somatic antigens* were prepared from 20-hour beef extract peptone broth cultures which had been heated at 100°C for 2½ hours. They were preserved by the addition of 0.4% formalin. *K type antigens* were prepared from living cultures grown for 12-17 hours on fresh beef extract agar slants containing 0.1% glucose. The slide agglutination technic was used for the demonstration of K type antigens. *H antigen* preparation was attended with some difficulty at the beginning of the study. It was discovered, however, that 3 or 4 rapid passages through a test tube column of semisolid agar 10 cm in depth (beef extract peptone broth with 0.2% agar) would produce motile cultures wholly satisfactory for agglutination tests and for immunization. Following motility enhancement cultures were inoculated into beef extract peptone broth and incubated for 16 hours. Cultures were killed and preserved by the addition of 0.4% formalin. In the preparation of *antiserums,* young rabbits weighing from 4 to 5 lb were given a series of 4 intravenous antigen injections in the amount of 0.5 cc, 1.0 cc, 1.5 cc, and 2.0 cc. Injections were given at 3- to 4-day intervals. One week after the last injection, the animals were bled. Serums were mixed with an equal volume of C. P. glycerol and stored. All serums were tested for the presence of alpha antibody by the employment of the Wakefield alpha strain. None of the serums used in this study contained alpha antibody.

Results. In our study of O antigens, 1:20 dilutions of the serum-glycerine mixture were made in 0.5% phenolized saline solution. These stock dilutions were the starting point for all titrations reported. Stock dilutions of seventeen O serums were prepared. Cross agglutination tests were conducted at a 1:40 dilution. Where cross agglutination was observed, titrations were started at 1:80 Table

TABLE I. Cross Agglutination of Heat Stable Antigens (2½ Hr at 100°C).

	Antiserums																
	ATC		Stuart's bio-type 32011									Stuart's Related bio-types				Ewing	
Antigens	962	222	SRC	68411	56211	63511	70811	C3	C5	C6	C7	37511	35611	37711	32821	2556	979
ATC 962	1280*	—†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ATC 222	—	1280	640	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SRC	—	640	640	—	—	—	—	—	—	—	—	—	—	—	—	—	—
68411	—	—	—	1280	—	—	—	—	—	—	—	—	—	—	—	—	—
56211	—	—	—	—	1280	—	—	—	—	—	—	—	—	—	—	—	—
63511	—	—	—	—	—	640	—	—	—	—	—	—	—	—	—	—	—
70811	—	—	—	—	—	—	2560	—	—	—	—	—	—	—	—	—	—
C3	—	—	—	—	—	—	—	640	—	—	—	—	—	—	—	—	—
C5	—	—	—	—	—	—	—	—	1280	—	—	—	—	—	—	—	—
C6	—	—	—	—	—	—	—	—	—	1280	640	—	—	—	—	—	—
C7	—	—	—	—	—	—	—	—	—	640	640	—	—	—	—	—	—
37511	—	—	—	—	—	—	—	—	—	—	—	2560	—	—	—	—	—
35611	—	—	—	—	—	—	—	—	—	—	—	—	1280	—	—	—	—
37711	—	—	—	—	—	—	—	—	—	—	—	—	—	640	640	1280	640
32821	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	1280	1280	1280
2556	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	1280
979	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	640	1280	640

* Figure indicates reciprocal of highest dilution at which a visible agglutination was observed by unaided eye.

† Minus (—) indicates no agglutination observed at 1:40 or higher dilutions.

I shows the O antigen relationships within the paracolon aerobacter-*A. cloacae* group. It appears obvious from the table that the cultures included in Stuart's biotype 32011 are by no means homogeneous in respect to their O antigens. On the contrary, among the 9 strains 8 completely different O antigens were demonstrated. Strains C6 and C7, however, proved to be identical. One of the 32011 cultures, SRC, proved to have the same O antigen as the *A. cloacae* culture ATC 222. The four strains, 37711, 32821, 2556, and 979 proved also to be identical in their O antigens. Antigen relationships, as stated were confirmed by reciprocal absorption tests. Culture ATC 962 proved to be unrelated to any of the paracolon Aerobacter cultures or to ATC 222.

H antigen relationships were investigated in a similar manner to that described for the thermostable somatic antigens. Seventeen H antiserums were prepared. Stock serum dilutions were made with 0.85% sodium chloride solution. Chloroform was added to the stock serum dilutions as a sterilizing and preserving agent(10). Cross titrations were performed beginning with a dilution of 1:40 and continuing to the titer of the serum. As will be observed in Table II the H antigens show a greater degree of interrelationship than was evident in the thermostable somatic antigens. One culture, 70811, gave no evidence of being related to any of the other cultures tested. The H antigens of cultures C6 and C7 appear to be identical and unrelated to the other cultures. ATC 222, ATC 962, and C3 also appear to be identical and unrelated to the other members of the group. The remaining eleven cultures show considerable H antigen relationship, but fall into three divisions as indicated by the enclosing brackets in Table II. The four strains of bracket 1 give cross reactions practically to the homologous titers of the serums not only with each other but also with the cultures of brackets 2 and 3. The 4 cultures of bracket 2 though giving good cross reactions with each other and with the bracket 1 strains, fail completely to cross with the 3 antigens of bracket 3. These latter give good reactions with the 4 members of bracket 1.

TABLE II. Cross Agglutination of H Antigens.

H antigens	H serums																
	1				2				3								
	SRC	37711	2556	979	68411	56211	63511	37511	35611	C5	32821	ATC 222	ATC 962	C3	C6	C7	70811
SRC	*5120*	2560	5120	2560	2560	2560	2560	5120	2560	2560	2560	—	—		—	—	—
37711	5120	*5120*	5120	5120	2560	2560	2560	2560	2560	2560	2560			—	—	—	—
2556	5120	5120	*5120*	5120	2560	2560	2560	2560	2560	2560	2560		—		—	—	—
979	5120	5120	5120	*5120*	2560	2560	2560	2560	2560	2560	2560				—		—
68411	2560	2560	2560	2560	*5120*	5120	5120	5120	—		—	—		—	—		—
56211	2560	2560	2560	2560	5120	*5120*	5120	5120	—	—	—	—	—	—	—	—	—
63511	5120	2560	2560	2560	5120	5120	*5120*	5120	—	—	—	—	—	—	—	—	—
37511	2560	2560	2560	2560	5120	5120	5120	*5120*	—		—	—	—	—	—	—	—
35611	2560	2560	2560	2560	—	—	—	—	*5120*	5120	5120	—		—	—	—	—
C5	2560	2560	2560	2560	—	—	—	—	5120	*5120*	5120	—	—	—	—	—	—
32821	2560	2560	2560	2560	—	—	—	—	5120	5120	*5120*	—	—	—	—	—	—
ATC 222	—	—	—	—	—	—	—	—	—	—	—	*5120*	2560	2560	—	—	—
ATC 962	—	—	—	—	—	—		—	—	—	—	2560	*5120*	5120	—	—	—
C3	—	—	—	—	—	—	—	—			—	5120	5120	*5120*	—	—	—
C6	—	—	—	—	—	—	—	—			—				*5120*	5120	—
C7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5120	*5120*	—
70811	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*5120*

TABLE III. Phase Variation of Culture 2556.

H antigen		H serum 2556 Un-absorbed	 Absorbed by 2556 (phase 1)	 Absorbed by 2556 (phase 2)
2556		5120	2560	2560
	phase 1	2560	—	2560
	" 2	2560	2560	—

TABLE IV. Phase Variation of ATC 222.

H antigens		H serum ATC 222 Unab-sorbed	 Absorbed by ATC 222 (phase 1)	 Absorbed by ATC 222 (phase 2)
ATC 222		5120	2560	2560
	phase 1	2560	—	2560
	" 2	2560	2560	—
	/962*	2560	—	2560
	/C3*	2560	—	2560
962		2560	—	2560
C3		2560	—	2560

* ATC 222/962, ATC 222/C3 indicates H phase of ATC 222 suppressed by serums 962 and C3.

These apparently anomalous results seem to be explainable only on the assumption that the strains of bracket 2 and those of bracket 3 are related each to a different H antigen of the cultures included in bracket 1. Such a relationship suggests the occurrence of phase variation in the flagellar antigens of bracket 1 cultures.

In an effort to reveal *phase variation* in the 4 cultures of bracket 1 H antigens were prepared from single colony isolations from culture 2556. These were used for absorbing 2556 antiserum. Tests with these absorbed serums indicated that the colonies were of two antigenic types in respect to their H antigens. Table III gives the results obtained with these absorbed serums and single colony antigens prepared from culture 2556. Subsequently culture 37711, SRC, and 979 and their homologous serums were similarily investigated with exactly the same results.

This related group of 11 cultures, therefore, appears to fall into 3 sub-groups. The first is composed of cultures SRC, 37711, 2556, and 979 (bracket 1) which are diphasic and identical to each other in both phases. The second sub-group (bracket 2) consists of cultures 68411, 56211, 63511, and 37511 which have H antigens identical to one of the phases occurring in bracket 1 cultures. Arbitrarily we shall call this antigen phase 1. The third sub-group, 35611, C5, and 32821 (bracket 3) contains an H antigen identical to that occurring in the other phase (phase 2) of the diphasic group.

Further investigations of the diphasic and monophasic cultures of this group were made employing the phase suppression technique. By this method it was found possible to obtain the desired phases from all of the diphasic cultures previously described. The results obtained were in every way similar to those given by the colony selection and serum absorption techniques mentioned above. These techniques were also applied to the remaining cultures of our series. Only one of these latter cultures, however, (culture ATC 222) was found to be diphasic. Cultures 962 and C3, appearing in Table II to be identical to ATC 222, were found to be monophasic and to possess only one of the H antigens of ATC 222. Table IV gives results demonstrating phase variation in culture ATC 222 and indicates the H antigenic relationships of that culture and cultures 962 and C3.

Cultures C6 and C7 were found to be identical, monophasic and unrelated to the other cultures. Culture 70811 proved to be monophasic also and unrelated to any of the other cultures studied.

TABLE V.
O and H Antigen Relationships Summarized.

Cultures	Thermostable antigens	H antigens
SRC	1	1:2*
ATC 222	1	3:4
ATC 962	2	3
68411	3	1
56211	4	1
63511	5	1
37511	6	1
C5	7	2
35611	8	2
32821	9	2
37711	9	1:2
2556	9	1:2
979	9	1:2
C3	10	3
C6	11	5
C7	11	5
70811	12	6

* This form is used to indicate the diphasic nature of the antigens. No implication is intended as to their group or specific nature.

The K antigens, characterized by their ability to inhibit O agglutination, include the thermolabile L and B envelope antigens and the capsular A type antigen. Our cultures were tested for these antigens by means of the slide agglutination technique using living cultures suspended in 0.85% sodium chloride solution. Varying dilutions of the homologous O serums were mixed with these bacterial suspensions and observed for evidence of agglutination. In all cases agglutination occurred normally. In fact in a few instances it appeared that living antigens were more sensitive to the O serums than the corresponding boiled antigens. These observations appeared to exclude the occurrence of the thermolabile L and B antigens in our cultures. To detect or rule out the A type antigens, however, requires a somewhat different technique, since antibodies against these thermostable antigens may be present in our O serums. Knipschildt(11) solved this problem by isolating from his A-containing coli cultures variant A minus forms. Later Vahlne(12) reported that some of his cultures isolated as A minus forms actually contained a small amount of A antigen, sufficient in fact, to cause inhibition of O agglutination. He further reported, however, that A antigen could be destroyed by heating at 120°C for 2 hours leaving the O antigens intact. We attempted both of these methods. We were unsuccessful in our attempts to isolate from our cultures colonies having the characteristics of Knipschildt's A minus forms. When autoclaved antigens were used to immunize animals we found that no demonstrable agglutinins appeared in the animals either for autoclaved or boiled antigens. The antigenic properties of our cultures appeared to be totally destroyed by this treatment. These results suggest the possibility that antigens in our boiled cultures (Table I) may be of the A type rather than true O antigens.

Discussion and conclusion. In a consideration of the experimental results reported here it appears obvious that the thermostable somatic antigens in this group of microorganisms are extremely heterogeneous. Seventeen cultures were examined and from this number, 12 somatic antigenic types were established. Nine cultures in this series represented the biotype 32011 and of this number eight contained distinct somatic antigens. Cultures C3, C5, C6, and C7 representing the 32011 type isolated from a gastroenteritis epidemic were found antigenically to belong in three distinct somatic groups. It seems improbable that the inciting cause of an epidemic would be represented by such a diversified group of antigenic types.

When one considers the flagellar antigens of this group it becomes apparent that Stuart's findings of near antigenic homogeneity in his 32011 cultures was in all probability due to the interrelationships of a few, but relatively commonly occurring, H antigens. The occurrence of phase variation in these antigens serves to increase the possibility of finding antigenic relationships in this group.

While a consideration of biochemical variants has been but a minor phase of this study, nevertheless it has been kept constantly in mind and especially in so far as it concerns lactose fermentation. No difficulty was encountered in isolating slow lactose fermenting variants from normal *A. cloacae* cultures when these were plated upon appropriate media. The obtaining of rapid lactose fermenters from slow fermenting cultures was attended with much more difficulty. Paracolon aerobacter cultures which have been isolated in the course of a search for enteric pathogens are normally selected because of their resemblance to non-lactose fermenting pathogens. Obviously colonies showing evidence of fermentation would not be isolated unless some special investigation was in progress. It would appear that it should now be possible to compare and determine by antigenic means whether lactose fermenting and non-lactose fermenting colonies obtained from a single individual are of the same antigenic type.

While no definite conclusions can be drawn from this study as to the relationships of paracolon aerobacter cultures of the biotype 32011 to normal *A. cloacae*, represented in this study by ATC 222 and 962, the experimental results indicate that these organisms have as much antigenic relationship to the normal forms as they have to each other. A definite conclusion in this matter awaits a more extensive investigation of the antigens contained in a larger group of normal *A. cloacae* cultures.

The present investigation has shown that it is not only possible but wholly feasible to classify paracolon aerobacter cultures of the *A. cloacae* type by means of antigenic analysis. It would appear premature to establish an antigenic schema for this group though data summarized in Table V suggest such a system could be devised.

In the future when organisms of the *A. cloacae* type are encountered, especially if associated with disease, it would seem desirable that such bacteria should be antigenically typed. Such a procedure should increase the probability of determining the pathogenic properties of this group.

Summary. 1. Paracolon aerobacters represented by biotype 32011 and related cultures were subjected to antigenic study and comparison. 2. A single biotype was found to be extremely heterogeneous in respect to its thermostable somatic antigens. 3. The occurrence of phase variation in the flagellar antigens of this group is reported and H antigenic relationships are determined. 4. It is pointed out that the antigenic system of classification can be profitably utilized in further investigation of this group.

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Heidenhain Pouch Secretory Response as Affected by Gastrojejunostomy to the Main Stomach.* (19813)

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Gastrojejunostomy has waned in popularity as a surgical procedure for the cure of peptic ulcer during the past 2 decades. Gastrojejunostomy was formerly advocated as a means of neutralizing the gastric secretion. We have not found any reports in the literature dealing with a quantitative study of the acid secretory response of the gastric mucosa to this procedure. We therefore undertook the following experiment.

Method. A Heidenhain pouch was produced and drained externally by a water tight nylon cannula in each of 5 healthy mongrel dogs. Following recovery from the anesthesia (intravenous Nembutal), the dogs were placed in individual cages in a special room. Fresh water was provided at all times. Milk and soft food were given for the first 3 or 4 postoperative days and, thereafter, the dogs were given a single daily feeding of 100 g cooked

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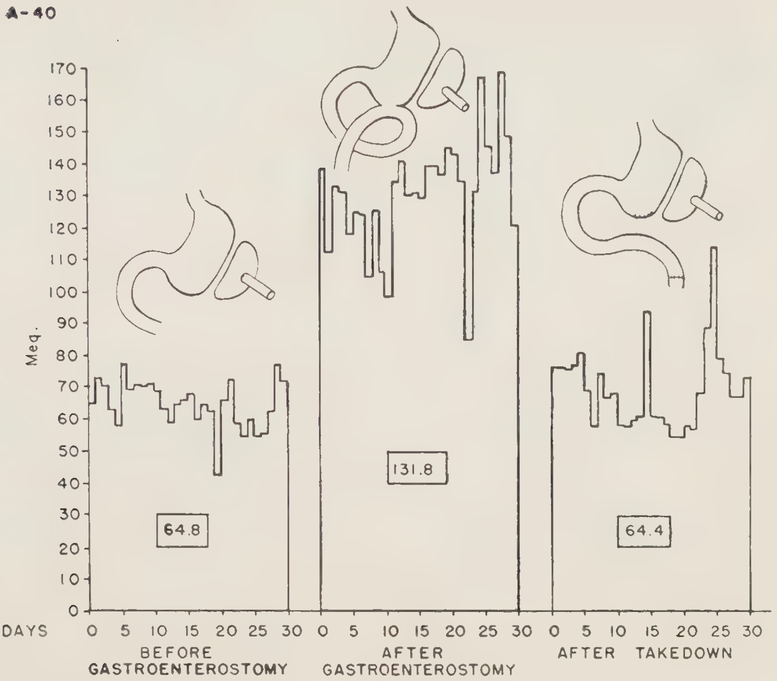

FIG. 1. Twenty-four hr free HCl output of a Heidenhain pouch in a representative dog during a 30-day control period and during similar periods following gastrojejunostomy to the main stomach and subsequent to take-down of the gastrojejunostomy.

horse meat, 500 g canned dog food, and 100 g condensed milk. A daily record was kept of the activity, general condition, appetite, zest, total food consumed, and regurgitation of food. Reingestion of regurgitated food was prevented by coarse mesh false bottoms in the cages. Twenty-four hour collections of pouch secretion were begun after full recovery from the operation—usually 2 or more weeks postoperatively. Rubber bags attached to the cannulas by threaded connectors were used for this purpose. Secretions were discarded if the animal was ill, refused food, or regurgitated. All specimens were examined, measured, titrated, and the data recorded daily. Body weights were recorded weekly. Total sodium and potassium contents of the secretion were determined by flame photometry at intervals and found to be well below the daily intake of these elements. After a continuous period of 4 weeks of constant collection, the animals were subjected to a short loop, antecolic, isoperistaltic gastrojejunostomy situated at the most dependent portion of the stomach, about 6 to 7 centimeters proximal to the pylorus, at about the upper margin of the antrum. The stoma was 4 cm in length. Collections were recommenced after recovery was complete as mentioned above, and the collection cycle repeated. Patency of the stoma was demonstrated radiologically after administration of barium sulfate. Two of these animals were subjected to take-down of the gastrojejunostomy after a month of collection and again secretory data were obtained for 30 or more days.

Results. A significant increase in free HCl output from the Heidenhain pouch occurred in each dog after gastrojejunostomy. This varied from 98% to 225% in individual animals, the average increase for the group being 142%. The free HCl output was proportional to the volume of secretion. There was no appreciable change in combined HCl output from the pouch. Gastrojejunostomy take-down in 2 of the dogs resulted in a reduction of HCl output from the pouch to the pre-gastrojejunostomy level in one dog as shown in Fig. 1, and in the other to a daily secretory level somewhat higher than the pre-gastro-

TABLE I. Average Daily Values of Free HCl Secretion from Heidenhain Pouches Expressed as mEq. Before and After Gastrojejunostomy to the Main Stomach and After Gastrojejunostomy Take-Down.

	mEq. free HCl/day				
Dog No.	Before gastrojejunostomy	After gastrojejunostomy	% rise	mEq. free HCl/day after gastrojejunostomy take-down	% drop
A-67	29.6	95.3	225	—	—
39	31.7	84.3	172	—	—
83	35.7	70.7	98	—	—
40	64.8	131.8	103	64.4	51
C-10	14.2	43.2	204	25.3	41
Avg	35.2	85.1	142	—	46

jejunostomy figure, but much lower than that obtained during the gastrojejunostomy period. These data are summarized in Table I

Discussion. The mechanisms responsible for this phenomenon are not definitely known to us at this time. We realize that the Heidenhain pouch differs from the normal stomach, but the former is an accurate indicator of the response of the parietal cells to humoral influences.

It is clear from the foregoing that gastrojejunostomy in some way is responsible for an increased output of HCl by the parietal cells. This could represent a compensatory response of these cells to intragastric neutralization of HCl to beyond physiologic limits —the "rebound phenomenon." Storer *et al.* (2) have shown that complete intragastric regurgitation of alkaline duodenal juices will cause a rise in Heidenhain pouch secretion of 100% or more. The possibility of increased antral stimulation due to repetitious passage of food through a normally patent pylorus and back into the stomach via the afferent loop of the gastrojejunostomy also may exist in some instances. Undoubtedly, in the main stomach the various neutralizing factors will tend to keep the concentration of free HCl at lower levels than obtain in the pouch. It seems reasonable to assume that the increased acid production may not always be neutralized and may play a part in the frequent failure of gastrojejunostomy as a treatment for peptic ulcer.

Conclusion. Under the conditions of this experiment, gastrojejunostomy of the main stomach of Heidenhain pouch dogs results in a significant rise in free HCl secretion from the pouch.

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Localization of the Enzymatic Block in Kidneys of Rats Treated with Fluoroacetate. (19814)

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Recent experiments have indicated that a "fluorotricarboxylic acid" formed from fluoroacetate inhibits each of the reactions of the aconitase enzyme *in vitro*(1). This finding would appear to explain the accumulation of citrate in the tissues of animals treated *in vivo* with fluoroacetate(2,3) as well as its accumulation and failure to be oxidized *in vitro* when fluoroacetate is present in reaction mixtures otherwise adequate for citrate oxida-

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TABLE I. Oxidation of Substrates by Homogenates of Kidneys of Rats Treated with Fluoroacetate Compared with Controls.

	60 min. total			1st 10 min.		
		μl O_2 (avg and range)			μl O_2 (avg, range)	
Substrate	No. of animals	FAc	Control	No. of animals	FAc	Control
Citrate	8	117 (66-144)	300 (254-357)	10	31 (7-45)	55 (34- 79)
Cis-aconitate	7	251 (177-275)	260 (207-323)	6	48 (13-73)	53 (34- 63)
α-ketoglutarate	2	318 (296-340)	358 (324-411)	3	68 (64-71)	78 (64- 93)
Pyruvate and fumarate	5	237 (188-284)	289 (226-325)	6	56 (44-66)	76 (64-100)

tion and synthesis(4,5). Nevertheless, it seemed desirable to compare the oxidative metabolism of citrate and other acids of the Krebs cycle *in vitro* following the administration of fluoroacetate *in vivo,* in an attempt to learn whether all of the reactions that are presumed to require the participation of aconitase were equally affected, since there was already evidence that aconitase was not blocked in some circumstances(2,6).

Materials and method. Dose of fluoroacetate. To establish the fluoroacetate block *in vivo,* male rats (weighing 200-300 g) obtained from the Holtzman-Rolfsmeyer Rat Co., were injected intraperitoneally with 5 mg of fluoroacetate per kg and sacrificed one hour later. Poisoning was evidenced by accumulation of citrate in the tissues(2,3) and/or convulsive states. *Oxidation studies.* The kidneys were excised and placed in ice-cold isotonic KCl solution; after weighing, they were homogenized in sufficient isotonic HCl to produce a 10% final homogenate, weight to volume. In addition to the basic medium reported previously(4), each flask contained substrates added as the potassium salt, brought to pH 7.2-7.4. Center wells contained 0.2 ml of 2.5 N NaOH. Cold homogenates were added to cold reaction mixtures and the vessels were equilibrated 10 min. before manometric readings were begun. The gas phase was air and the temperature was 38°C. Reactions were stopped by addition of 0.3 ml of 50% perchloric acid to the main chamber. Citrate determinations on the protein free filtrates were carried out by the method of Natelson *et al.*(7). *Aconitase studies.* 40 μM of cis-aconitate were added to the solution containing the homogenate preparation, cysteine, and ferrous ammonium sulfate at the concentrations recommended by Dickman and Cloutier(8). After 10 min. of incubation at 30°, sufficient 20% trichloroacetic acid was added to produce a final concentration of 5%. Citrate was determined as noted above. *Aconitase and oxidations.* To test both the aconitase and oxidative functions simultaneously, 20 μM of cis-aconitate were added to flasks containing kidney homogenates, basic medium and sufficient arsenite to depress α-ketoglutarate oxidation, yet not depress oxygen uptake (0.0002M). At various time intervals, oxygen uptake, α-ketoglutarate formation and citrate formation were measured.

Results. The data of Table I indicate that oxidation of all added substrates was significantly less in flasks containing homogenates of poisoned kidneys as compared with the controls, but the greatest effect observed was on oxidation of citrate. Not only was the initial oxidation of citrate depressed to 45% of the control, but the total oxidation was only 32% of the control. By contrast, the oxidation of cis-aconitate and α-ketoglutarate were respectively 96% and 89% of the control values for the total period of incubation, while the initial oxidation was 91% and 87% of the control. In agreement with previous findings of inhibition of oxidation reactions prior to the level of citrate in the Krebs cycle, it was found that oxidation of pyruvate and fumarate was 82% of the control for the total period while oxidation in the first 10 minute interval was 74% of the control.

As in previous investigations(2,6), the aconitase activity of these preparations was not diminished, as measured either by conversion of cis-aconitate to citrate or conversion of isocitrate to citrate.

It seemed possible that the failure of citrate oxidation could be related to defects in hydrogen transport, or a relative excess of

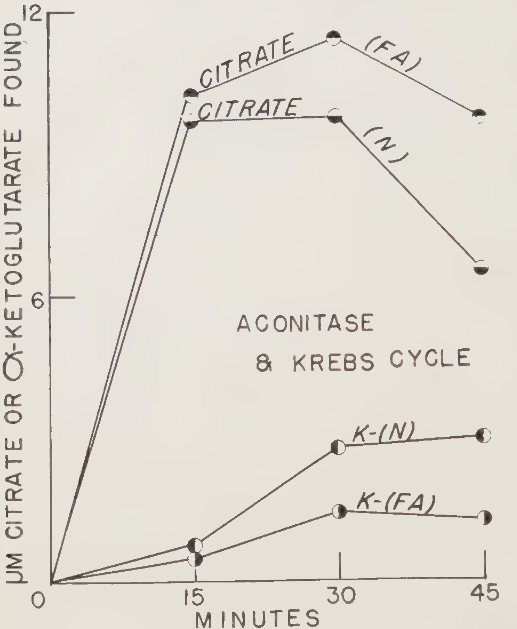

FIG. 1. Simultaneous conversion of cis-aconitate to citrate and oxidation of cis-aconitate. Conditions as in text, but flasks contained 60 mg kidney tissue from normal (N) or fluoroacetate-treated (FA) rats and 5 μM ATP in these experiments. Figures presented are avg for two experiments each performed in duplicate.

dephosphorylation. To test this point, experiments were carried out with cis-aconitate as the substrate and citrate formation and disappearance was measured. As Fig. 1 shows, the conversion of cis-aconitate to citrate was the same in both control and fluoroacetate systems; yet the latter failed to reconvert the citrate pool during the course of the incubation period. The result was obtained over a time period where the rate of oxidation was essentially the same in both homogenates. It appears that there is no defect in phosphate transfer or hydrogen transport.

The data from this experiment as well as the data from Table I show that in kidney homogenates from fluoroacetate-treated animals there is a marked decrease in the ability to convert citrate to cis-aconitate with little if any diminution in the ability to carry out the reverse reaction. These findings are in agreement with those of Elliott and Kalnitsky(5) and of Lotspeich, Peters, and Wilson(1) who used different experimental approaches to this problem, and show that the main block occurs at the citrate to aconitate step. These findings might be explained if the dissociation constant for cis-aconitate and aconitase is small enough compared to that of citrate to permit it to compete effectively with the fluoroacetate derivative under conditions in which citrate could not compete(1). Alternatively, one would have to seek additional mechanisms for interconverting the two compounds(5).

Discussion. In consideration of the physiological effects produced by fluoroacetate, it is evident that in the affected cell, a particularly vulnerable site in energy metabolism is attacked. Essentially, progress at the tricarboxylic acid level is effectively blocked, inasmuch as such little cis-aconitate or isocitrate as could be mobilized from sources other than citrate would be converted to citrate. Maintenance of the energy requirements of the blocked cell is then dependent upon conversion of glutamate and aspartate to components of the Krebs cycle and further oxidation of the α-ketoglutarate and oxalacetate formed; since the block varies from tissue to tissue in the extent of its completeness, the citrate accumulated may also serve in part to overcome the effects of the block. It seems possible that the symptomatic response to fluoroacetate poisoning will occur in tissues which have a high energy demand and a low capacity for conversion of amino acids to substrates of the Krebs cycle. The brain, having a great demand for energy from oxidations, and a low capacity for protein breakdown may be such an organ.

Summary. 1. Oxidation of citrate was markedly depressed in homogenates of kidneys from rats treated with fluoroacetate; oxidation of other substrates of the Krebs cycle, including cis-aconitate, was depressed only to a relatively small extent. 2. No inhibition of aconitase was found in these preparations when cis-aconitate was the substrate. 3. The evidence indicated that the conversion of cis-aconitate to citrate was relatively unaffected under conditions in which the reverse reaction was strongly inhibited.

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The Antibacterial Action of Erythromycin.* (19815)

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Erythromycin is a new antibiotic obtained from *Streptomyces erythreus*. Some features of the isolation and production of this antibiotic and of its physical, chemical and biologic properties were described briefly by McGuire *et al.*(1). They found erythromycin to be active *in vitro* against a wide variety of microorganisms, including strains of penicillin-sensitive and penicillin-resistant staphylococci, and of relatively low toxicity both for laboratory animals and in preliminary trials in patients. The prospect of a new agent that is active and nontoxic and which might prove to be effective against staphylococcal infections prompted us to undertake further studies on erythromycin. A preliminary clinical report has been published elsewhere(2) and the results of the laboratory findings are presented in this and the succeeding 2 papers. Some laboratory and clinical observations have also been presented by Heilman *et al.*(3).

Materials and methods. Erythromycin. A supply of "Ilotycin" (Erythromycin, Lilly) for clinical trial and of the purified crystalline material (890 μg/mg) for laboratory studies was furnished by the Lilly Research Laboratories through the courtesy of Dr. J. W. Smith. Stock solutions of the white crystalline powder were made either in broth or distilled water so as to contain 8 mg or 2 mg of the free base per ml; a small amount of ethyl alcohol was used to facilitate solution. Further dilutions of the antibiotic were made in broth unless otherwise indicated. *Bacterial Strains.* Most of the strains of organisms used in this study were recently isolated from infected material obtained from patients at the Boston City Hospital. Some of them were isolated and identified by Marion E. Lamb and A. Kathleen Daily in the Bacteriological Laboratory of the Mallory Institute of Pathology; the strains of gonococci were isolated by Helen W. Trousdale in the Genito-Urinary Clinic and most of the other organisms were isolated by Clare Wilcox and Marilyn K. Broderick to whom we are also indebted for technical assistance throughout this study. Most of the strains of specific groups and types of hemolytic streptococci were obtained from Drs. Rebecca Lancefield, Lowell A. Rantz and Harry A. Feldman. The strains of *Streptococcus mitis, sanguis* and *faecalis* from cases of bacterial endocarditis were obtained from Dr. Leo Loewe. In addition, a few laboratory strains used routinely for assay of other antibiotics were also included. For routine tests of sensitivity, the cultures used were obtained by picking individual colonies from the surface growth of culture plates; however, in the studies on the development of resistance and on the mechanism of action of the antibiotic, individual colonies were picked from agar pour plates of the cultures which had been so diluted as to contain only a few well segregated colonies. *Sensitivity Tests.* These were carried out with serial 2-fold dilutions of the

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TABLE I. Stability of Erythromycin in Broth or Serum at Various Temperatures.

Temp., °C	Solvent	Assayed erythromycin concentration, μg/ml Days of storage 0	1	2	4	7	14	21	28	44	56
–25 5	Broth	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
22–30	”	2000	2000	2000	2000	1000	2000	1000	1000	1000	1000
37	”	2000	2000	2000	1000	1000	1000	1000	500	250	125
–25	”	2	2	2	2	2	2	2	1	2	2
5 22–30	”	2	2	2	2	1	1	1	1	1	1
37	”	2	2	2	2	1	.5	.5	.25	.25	.13
–25 5	Serum*	2000	2000	2000	2000	2000	2000	2000	2000		
37	”	2000	2000	2000	1000	1000	1000	500	250		
–25 5	”	2	2	2	2	2	2	2	2		
37	”	2	2	2	1	.5	.5	.25	.13		

Final pH of the 2000 μg solution in broth was 7.7, that of the 2 μg solution was 7.5. *Sarcina lutea* in a dilution of 10^{-4} was used as the assay organism.

* 50% pooled human serums.

TABLE II. Effect of Heat and Filtration on Activity of Erythromycin in Broth.

Treatment	Temp., °C	Duration	Assayed conc. of erythromycin, μg/ml Before	After	Before	After
Heated	60	5 min.	200	100	2	1
		15				
		1 hr				
		2	”	50	”	”
Heated	100	5 min.				
		15				
		1 hr	”	”	”	.5
		2	”	12.5	”	.125
Seitz filtered			”	50	”	1
Berkefeld filtered			”	”	”	”
Sintered glass filtered			”	100*	”	” *
None—control			”	200	”	2

Streptococcus 98 diluted 10^{-4} used for assays in broth.

* Partial inhibition in twice this concentration.

antibiotic, either in broth (Brain-Heart Infusion Broth, Difco, pH 7.4±) or in agar (Heart-Infusion Agar, Difco, pH 7.4±). Defibrinated horse blood, 0.5%, was added to the broth to serve as an indicator of growth and 10% was added to the agar for use with hemolytic or fastidious organisms. The inoculum in the broth-dilution test was such as to make a final dilution of 10^{-4} of the seed culture; in the agar-plate dilution test a 2 mm loopful of the undiluted culture was used. The sensitivity of a strain is expressed as the final concentration of antibiotic in the medium that completely inhibited growth in 18-24 hours as judged by visible turbidity or hemolysis in the broth or by the appearance of growth or hemolysis on the agar plates. Growth inhibition in broth was verified by subculture on antibiotic-free agar.

Observations on Antibacterial Properties of Erythromycin. Effect of Storage. Solutions containing 2000 and 2 μg/ml in broth were stored as follows: a) at –25°C in a "deep freeze," b) at 5°C in a refrigerator, c) at 22-30°C on a laboratory shelf and d) at 37°C in an incubator. Similar solutions in 50% pooled human serum were also stored in like manner, but omitting room temperature. As shown in Table I, all of the solutions retained their activity remarkably well at –25°C throughout the periods of observation. The same was true at 5°C, except for a detectable

TABLE III. Effect of pH of Medium on Sensitivity of Bacteria to Erythromycin.

pH of broth	Min complete inhibiting conc. of erythromycin, μg/ml *Strep.* 98	*S. lutea*
5.58	1.6	.8
6.05	.8	.4
6.51	.2	.1
7.08	.04	.04
7.46	.02	.01
8.07	.01	.003
8.53	.003	.001
	Growth without antibiotic	
5.58	+	++
6.05	++	+++
6.51 7.08 7.46	+++	+++
8.07	+++	++
8.53	++	+

drop in activity of the 2 μg solution in broth by the end of the first week without further deterioration thereafter. At room temperature there was a similar loss of activity in broth at both concentrations, while at 37°C there was progressive deterioration in activity of all the solutions after 4 or 7 days.

Effect of heating. As shown in Table II, there was some loss of activity detected even after 5 minutes at 60°C; the loss appeared to be greater from the higher concentration and after longer exposures, particularly at 100°C.

Effect of Filtration. (lower part of Table II). Passage through a Seitz filter pad (Hercules, type ST, size L3) or through a Berkefeld filter entailed a detectable loss of activity from a concentration of 2 μg and a 75% loss from a 200 μg/ml solution in broth. In solutions passed through sintered glass filters (Corning UF) there was also a slight loss. All of the filters, except the Seitz filter pads, had previously been used several times for other materials.

Effect of pH. The erythromycin sensitivity of the test strains *Strep.* 98 and *S. lutea* were determined in broth adjusted to various pH levels ranging from about 5.6 to 8.5. As shown in Table III, the sensitivity of both strains increased progressively with increasing alkalinity of the medium; the increase was roughly 10-fold for each pH unit. Growth at the extreme pH values was poor even without antibiotic, but the inhibition by erythromycin was clearly discernible. This effect of pH is similar to that observed with streptomycin(4) and is the reverse of that noted with aureomycin(5).

Effect of Various Substances. The following substances, in the final concentrations indicated, were used in heart infusion broth for assay of the erythromycin sensitivity of *Bacillus cereus*, No. 5:

Sodium chloride Dextrose	M/100, M/10, 1M
Sodium thioglycollate Cysteine hydrochloride Semicarbazide Urea Glutamic acid	M/1000, M/100, M/10
Para-aminobenzoic acid	M/1000, M/500, M/100
Pteroylglutamic acid	M/1000, M/100, M/50

The following substances were similarly used with *Streptococcus* 98 as the test organism: the sodium salts of citric, pyruvic, acetic, lactic, fumaric and succinic acid, each in concentrations of M/100, M/10 and 1M. Penicillinase, 1000, 100, and 10 units, was also used with this organism. Pooled human serum was added to broth in concentrations of 12.5, 25, and 50% and assayed with *S. lutea* as the test strain. None of these substances, in the concentrations used, had any significant effect on the antibiotic activity of erythromycin against these respective test organisms in the crude test here employed.

Effect of Reduced Oxygen Tension. Two sets of blood-agar plates containing serial 2-fold dilutions of erythromycin were prepared and segments of each were inoculated with 2 strains of *Streptococcus* (Nos. C203 and 98), and one each of *S. lutea,* pneumococcus type 3, *Streptococcus viridans,* enterococcus and staphylococcus (No. 195). One set of these plates was then incubated at 37°C on an open shelf; the other was placed in a tightly sealed jar from which the oxygen was partially removed and CO_2 tension increased by burning a candle inside to extinction and the jar was then placed in the same incubator. The end-points of inhibition by erythromycin were identical for each organism in the 2 sets of plates. However, on the plates with the highest concentrations of antibiotic on which growth still occurred, that growth in the case of 4 of the strains was heavier after 24 hours in the candle jar than on the open shelf. End-points of inhibition by erythromycin were also found to be the same in brain heart infusion broth and in thioglycollate broth.

Erythromycinase. An attempt was made to determine whether erythromycin-inhibiting substances are produced during growth by organisms which are relatively resistant to that agent. In one series of studies the method used was essentially the same as the modified Gots test for penicillinase(6). A strain of *S. lutea* was employed; its growth was completely inhibited on the surface of agar containing 0.02 μg but not by 0.01 μg of erythromycin per ml. Plates were poured with agar containing both a 1:100 dilution of a fully grown culture of this strain and erythromycin in a final concentration of 0.02 μg/ml. The agar was allowed to harden rapidly in the cold and a number of strains of organisms were streaked radially on segments of these plates. The following organisms were used: 13 strains of *Pseudomonas aeruginosa,* 6 of *Aerobacter aerogenes,* 5 of *Klebsiella pneumoniae,* 7 of *Escherichia coli,* 4 of *Proteus vulgaris,* 2 species of *Salmonella* and 2 species of *Shigella.* In plate-sensitivity tests, all of these strains grew well on agar containing 200 μg/ml of erythromycin except the strains of *Salmonella* and *Shigella,* growth of the former being inhibited by 200, and of the latter by 100 μg/ml. All of the strains grew rapidly and luxuriantly on the surface of the agar containing the Sarcina and erythromycin. In no instance, however, was growth of colonies of Sarcina made out within the agar in the vicinity of the surface growths, the underlying and surrounding agar remaining perfectly clear as did the rest of the agar. This was taken to indicate that no erythromycin-inhibiting substances had diffused from the surface growth of any of the strains into the medium, at least before the erythromycin contained in that agar had either killed or completely inhibited the growth of the Sarcina. In a second type of experiment, there was no indication that the growth of *P. vulgaris* or *E. coli* in solutions of erythromycin for periods up to 72 hours had inactivated any of the antibiotic.

TABLE IV. Effect of Size of Inoculum on Erythromycin Sensitivity.

	M.I.C.,† μg/ml		Growth without antibiotic	
Size of inoculum*	Broth dilution	Agar-plate dilution	Broth	Agar
	Streptococcus 98			
Undiluted	—	.1	—	+++
10^{-1}	1.6	.04	+++	+++
10^{-2}	.2	.02	+++	+++
10^{-3}	.04	.02	+++	+++
10^{-4}	.02	.02	+++	+++
10^{-5}	.02	.01	+++	5 colonies
10^{-6}	.02	—	+++	—
	Sarcina lutea			
Undiluted	—	.02	—	+++
10^{-1}	.04	.02	+++	+++
10^{-2}	.04	.02	+++	+++
10^{-3}	.04	.02	+++	++
10^{-4}	.02	.01	+++	+
10^{-5}	.02	<.005	+++	1 colony

* Final dilution of culture in the broth-dilution test and the dilution from which a 2 mm loopful was streaked on each plate in the agar-plate dilution test. Pour plate counts of the original 18-hr cultures yielded approximately 10^8 colonies per ml of *Strep.* 98 and 2×10^7 colonies per ml of *S. lutea.*

† Minimum complete inhibiting concentration.

— = Not done.

Effect of Size of Inoculum. Streptococcus 98 and a strain of *S. lutea* were tested for sensitivity to erythromycin by both the broth- and the agar-dilution methods using inocula of varying sizes. The results are shown in Table IV. In these tests the strains appeared to be more sensitive when a small inoculum was used; the differences were greater with *Strep.* 98 than with *S. lutea.* However, there were considerable ranges of concentrations of culture in each test and with each strain over which the same end-points were obtained. Similar effects have been noted with other antibiotics(7). From a practical point of view, these results indicate that when tests for sensitivity to erythromycin are carried out in the routine manner employed here, that is, with the culture diluted to 10^{-4} in broth, or used undiluted or up to 1:10 on agar, the value obtained for the sensitivity of an organism may be the same by the two tests (as in the case of *S. lutea*) or the plate-dilution value may be higher (as with Strep. 98).

Comparison of Agar- and Broth-Dilution Methods for Determining Sensitivity to Erythromycin. It has been shown(7) that values for sensitivity of any given strain to a number

TABLE V. Comparison of Results of Erythromycin Sensitivity Tests Done by Broth-Dilution and Agar-Dilution Methods.

Organism	Min inhibiting conc., μg/ml						M.I.C. on agar / M.I.C. in broth	
	On agar		Complete / Partial	In broth		Complete / Partial		
	Complete	Partial		Complete	Partial		Complete	Partial
Streptococcus, C203	.04	.02	2	.02	.01*	2	2	2
98	.04	.02	2	.02	.01*	2	2	2
Bacillus cereus, 5	.4	.1	4	.2	.1	2	2	1
S. lutea	.02	.01	2	.02	.02	1	1	½
Staph. aureus, 195	.4	.2	2	.4	.2	2	1	1
192	.4	.2	2	.4	.2	2	1	1
193	.8	.2	4	.8	.2	4	1	1
194	.8	.2	4	.4	.1	4	2	2
197	.8	.2	4	.4	.1	4	2	2
Kl. pneumoniae, T	100	12.5	8	25	25	1	4	½
E. coli, MacLeod	100	12.5	8	12.5	6.3*	2	8	2
14	100	12.5	8	6.3*	6.3	2	16	2
15	100	12.5	8	6.3	6.3	2	16	2
Ps. aeruginosa, 5	>200	200		>200	>200			
6								
8								

All these results were recorded at 24 hr; they were identical after 48 hr in every instance when done by the agar plate method.

* In the tests done in broth, however, the values indicated by the asterisks (*) were twice as high after 48 hr.

of antibiotics may vary within wide limits depending on the details of the method used. The standard strains employed in antibiotic assays in this laboratory and a few additional strains were tested by both methods simultaneously, the same cultures and dilutions of antibiotic being employed for all tests. The results are shown in Table V. In these tests the value for "partial" inhibition indicates the minimum concentration of antibiotic with which there was significantly less growth than that observed in the corresponding culture without antibiotic. Only the readings made after 24 hours of incubation are shown in Table V, but in the case of the tests done on agar, identical readings were obtained at 48 hours in every instance. The same was true of the values for complete inhibition in broth except for one strain; in the tests in broth, however, there were a few strains in which the minimum concentration of erythromycin showing partial inhibition was one dilution higher at 48 hours than that recorded at 24 hours. The ratio of complete to partial inhibiting concentrations may be taken as an indication of the sharpness of the end-point of the test. In broth, this ratio was usually 2 (*i.e.*, only one 2-fold dilution tube showed partial inhibition), although for 3 of the strains of staphylococcus this ratio was 4 (*i.e.*, 2 tubes showed partial inhibition). On agar, the results were essentially the same except that the strains of *E. coli* and that of *Kl. pneumoniae* showed an 8-fold difference. This may be due to the fact that minor degrees of growth are more readily discernible with the strains on the agar plates than by grossly visible turbidity in broth. As a result, the values for sensitivity obtained for these coliform organisms, as measured by complete inhibiting concentrations were 4 to 16 times higher by the agar method than by the broth method. For the Gram-positive strains, the values obtained by both methods were either the same or varied by only 1 dilution regardless of whether partial or complete inhibition was chosen for the end-point. Because of its greater simplicity, the agar plate dilution method was, therefore, adopted for tests involving large numbers of cultures, it being appreciated that for coliform organisms (and possibly for others, as noted in the section dealing with size of inoculum) the values for sensitivity by this method may be appreciably higher than those obtainable in the broth-dilution method.

Sensitivity of Pathogenic Bacteria to Erythromycin. Tests for sensitivity to erythromycin were done by the agar plate-dilution method on 1037 strains of pathogenic bacteria,

TABLE VI. Sensitivity of 1037 Strains of Bacteria to Erythromycin.

		Min complete inhibiting conc./M.I.C., µg/ml									
A. "Sensitive" organisms	No. tested	3.1	1.6	.8	.4	.2	.1	.04	.02	.01	M.I.C. for ⅔ of strains, µg/ml
Pneumococcus, various types	64						6	29	27	2	.04
Streptococcus hemolyticus:											
A	58					1	7	30	20		.04
B	14				3	7	2	2			.2
C	21			2	4	8	5	2			.2
Group D	30	6	7	4	3	2	3	2	3		1.6
E	2						1	1			—
F	4							2	2		.04
G	20					6	7	4	2	1	.1
Endocarditis strains:											
Alpha streptococcus	12	1	2	1	1	2	2	1	2		.4
Streptococcus mitis	3	2					1				3.1
sanguis	7						2	2	3		.04
faecalis	4	2	2								3.1
Staphylococcus aureus	640		6	28	529	60	11	1	2	3	.4
albus	10	1	3	3	2	1					1.6
Corynebacterium diphtheriae	14	1	4	4	4	1					1.6
Neisseria gonorrhoea	10				3	4	1	2			.2
meningitidis	8		1	1	3	3					.4
Hemophilus influenzae	18	3	9	5	1						1.6
hemolyticus	10	2	4	2	2						1.6

		M.I.C., µg/ml				
B. "Resistant" organisms		>200	200	100	50	
Escherichia coli	17		5	8	4	100
Aerobacter aerogenes	12	2	10			200
Klebsiella pneumoniae	12	9	3			>200
Proteus vulgaris	8	8				>200
Pseudomonas aeruginosa	29	29				>200
Shigella flexneri	2	2				—
dysenteriae	1			1		—
Salmonella typhosa	4	2	1	1		>200
typhimurium	2	2				—
derby	1	1				—

most of them recently isolated from human sources. The results are shown in Table VI. All of the strains of Gram-positive cocci, *Neisseria* and *Hemophilus* were completely inhibited by 3.1 µg/ml or less and are listed as "sensitive"; the strains of coliform and enteric organisms all required 50 µg/ml or more and are listed as "resistant." The most sensitive were the pneumococci and group A hemolytic streptococci, while the most resistant of the organisms tested were the strains of *Proteus* and *Pseudomonas*. There were variations in sensitivity among strains of the same species. In the case of most of the species studied, however, these variations were confined to a 10-fold range of concentrations with more than three-fourths of the strains of each species being inhibited within a 4-fold range. The most divergent in sensitivity were the strains of group D streptococci and alpha hemolytic streptococci which were inhibited by concentrations varying from 0.02 to 3.1 µg/ml and those of *Staphylococcus aureus* which varied in sensitivity from 0.01 to 1.6 µg/ml. In the case of the streptococci, however, the strains were irregularly distributed throughout this range, whereas by far the greatest majority of the strains of *Staph. aureus* (87%) were inhibited by 0.4 µg/ml and 96% of them were inhibited by 0.2-0.8 µg/ml.

Comparison with Other Antibiotics. The great majority of the strains listed in Table VI have also been tested for sensitivity to the other antibiotics that are in common use, particularly to penicillin, streptomycin, aureo-

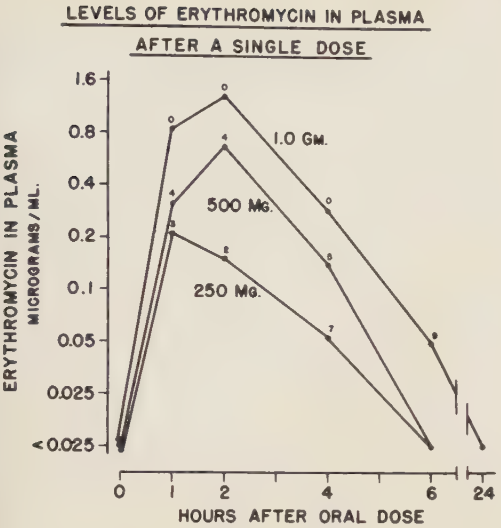

FIG. 1. Each point represents the mean for 15 samples. No. above each point indicates samples with $<.025$ γ/ml.

mycin, chloramphenicol and terramycin. On a weight basis, erythromycin most closely resembled penicillin in its activity; it was appreciably more active against Gram-positive organisms and less active against coliform and enteric bacilli than were the broad-spectrum antibiotics or streptomycin. Erythromycin was comparable with all of these agents in its inhibition of strains of *Hemophilus.* The majority of the strains of *Staph. aureus* included in Table VI were markedly resistant to penicillin, but erythromycin was equally active against both the penicillin-sensitive and the penicillin-resistant strains. Streptomycin-resistant organisms were also included in the tests and these likewise were as sensitive to erythromycin as were streptomycin-sensitive strains of the same species.

Absorption and Excretion. Single doses of 250 mg, 500 mg and 1.0 g were given by mouth to adults who had no recent antimicrobial therapy. Each dose was given to 15 subjects of varying ages and weights but selected so that the groups receiving the different doses were comparable in these respects. Specimens of citrated venous blood were collected before and at 1, 2, 4, 6 and 24 hours after the dose and the plasma was assayed for erythromycin by the 2-fold tube-dilution method, using *Strep.* 98 as the assay organism. The minimum assayable concentration in these tests was 0.025 μg/ml. None of the initial plasma specimens showed any erythromycin activity. The levels obtained after the single doses varied considerably in the individual patients, but in general higher concentrations were obtained more frequently after the larger doses. One subject who received 250 mg and 2 who got 500 mg failed to yield assayable erythromycin activity in any of the specimens and another patient who was given the smaller dose had a minimum demonstrable level only in the 2-hour specimen. In the specimens obtained at 6 hours, there was no demonstrable erythromycin in any specimen from patients who received 250 or 500 mg nor from 9 of those who received 1.0 g.

The maximum plasma concentration of erythromycin in each patient was found either at 1 or 2 hours after the dose; it was found in the 1-hour specimen most frequently after the smallest dose and in the 2-hour specimen after the larger doses. The mean concentrations of erythromycin at various intervals after each of the doses are charted in Fig. 1, which also shows the numbers of subjects who failed to yield assayable levels.

Plasma concentrations of erythromycin were also studied in patients receiving repeated doses of the antibiotic for therapy of infections. The results are shown in Table VII. Although some specimens contained no demonstrable antibiotic after repeated doses of 100 or 125 mg, in patients treated with doses of 200 mg or more every 3 or 4 hours erythromycin activity could be demonstrated in the plasma throughout the intervals between the doses. In general the concentrations varied with the dose and, in most patients, with the interval after the dose. Assays were also made of erythromycin in the urine of patients under treatment with this agent. In 6 patients who received a single dose of 0.25-1.0 g, the urine collected during the ensuing 24 hours showed no demonstrable activity in 5; a mean concentration of 0.04 μg/ml and a total of 60 μg was found in the 24 hour specimen from the remaining patient who had received 1.0 g. In several subjects who were receiving 100-200 mg every 4 hours, no erythromycin activity was demonstrable in the urine during the first

TABLE VII. Concentrations of Erythromycin in Plasma of Patients During Treatment.

Dose	Day of Rx	Erythromycin in plasma, μg/ml Hr after dose			
		1	2	3	4
100 mg every 3 hr	1	0*	0	0	
	2	0	0	0	
	2	.4	3.1	.4	
	3	0	0	0	
	3	0	.2	0	
	4	0	.4	0	
	6	0	.4	0	
	6	0	.4	0	
125 mg every 3 hr	3	.1	0	0	
	3	.4	.2	0	
	4	0	.2	0	
200 mg every 3 hr	3	0	.2	.1	
	3	.2	.2	.2	
	5	.2	.4	.2	
250 mg every 4 hr	3	.8	.8	.8	.2
	4	3.1	.4	.2	.1
	4	6.3	3.1	.8	.2
	5	1.6	.8	.4	.2
	5	.8	3.1	.8	.4
	6	3.1	1.6	.2	.2
	6	.8	1.6	1.6	.2
500 mg every 3 hr	5	12.5	6.3	3.1	
	6	25	6.3	3.1	
	6	6.3	3.1	1.6	
	7	3.1	3.1	1.6	
	10	12.5	6.3	6.3	
	11	6.3	3.1	3.1	
	17	6.3	25	3.1	

* 0 = <.025 μg/ml.

4 days of therapy. In those receiving 500 mg every 3 hours concentrations ranging from 50-800 μg/ml of urine were found in different specimens. Assays of total 24-hour urinary outputs were made at various intervals during the course of treatment of a number of patients; amounts of erythromycin varying from 0.02-15% of the daily amount ingested were recovered in different subjects. The greatest amounts were recovered during the second and third week of therapy from patients receiving doses of 500 mg every 3 hours. In a patient with staphylococcal meningitis under treatment with 500 mg of erythromycin every 3 hours, 5 specimens of spinal fluid were obtained and assayed at different times; concentrations varying from 0.1 to 0.8 μg/ml were found. The plasma obtained simultaneously was found to have 0.4 to 12.5 μg/ml, or from 4 to 16 times the concentration of the corresponding spinal fluid specimens.

Discussion. The data presented indicate that erythromycin has many of the physical, biochemical and biological properties of a useful antibiotic. The range of bacteria against which this agent is active is very similar to that of penicillin and is also embraced by the "broad-spectrum" antibiotics. In preliminary clinical trials(1-3) erythromycin appears to be effective against simple infections with Gram-positive cocci. However, the ready emergence of highly resistant variants among some of the organisms in the present studies and the clinical counterpart already observed in cases of bacterial endocarditis (1, 3) indicate that erythromycin will not be effective against all infections with these and other organisms, even if they do prove to be highly sensitive before treatment is begun. Failures may, therefore, be expected in situations where reasonably rapid sterilization of the lesion is not achieved.

However, there are many circumstances, such as hypersensitivity or untoward reactions of the patient to other effective antimicrobial agents, or resistance of the specific inciting strains to the other agents, in which erythromycin may prove to be very useful. Moreover, studies now in progress in this laboratory (by Drs. S. S. Wright and E. M. Purcell) indicate that the emergence of resistance to both erythromycin and streptomycin can be greatly delayed in organisms which are originally sensitive to both of these agents and are repeatedly subcultured in graded concentrations of both of them used in combination. These findings suggest that judiciously chosen and appropriately applied therapy with other antimicrobial substances in combination with erythromycin may extend the usefulness of the latter in the treatment of some important infections, for example those due to staphylococci which are resistant to many other agents.

Summary and Conclusions. The results of studies on some properties of erythromycin which may be important in its clinical and laboratory applications have been reported. 1. Erythromycin solutions retained their activity after prolonged storage in the cold or in the frozen state but showed progressive deterioration after several days at room or incubator temperatures or after brief exposures to 60°C or higher. 2. Filtration of erythromycin through bacterial filters entailed the loss of some activity. 3. The antibacterial action of erythromycin increased progressively with increasing alkalinity of the culture medium, within the pH range of bacterial growth. 4. Many substances which may affect the action of other antimicrobial agents had no important effect on the action of erythromycin. 5. Erythromycin inhibiting substances could not be demonstrated in cultures of erythromycin-resistant bacteria. 6. The size of the inoculum affected the results of sensitivity tests with erythromycin, but there was considerable tolerance within the range of inocula used in clinical testing. 7. The broth-dilution and agar-plate dilution methods generally gave comparable results in tests for sensitivity of bacteria to erythromycin but the values obtained by the agar method often were higher, particularly with some coliform organisms. 8. Tests done on more than 1000 bacterial strains, most of them recently isolated from patients, indicated that erythromycin was most active against the Gram-positive cocci and was quite active against strains of *Neisseria,* diphtheria bacilli and *Hemophilus,* but for practical purposes it could be considered inactive against most coliform and enteric bacilli. 9. Concentrations of erythromycin in plasma after single oral doses varied widely but in general were proportional to the dose. Maximum concentrations were found 1 or 2 hours after a dose, and no activity was demonstrated at 6 hours except following doses of 1.0 g. Significant concentrations were maintained in the plasma with oral doses of 250 mg or more every 3 or 4 hours. 10. The amounts of erythromycin activity recovered in the urine appeared to be small and erratic after ingestion of single doses or after repeated small doses, particularly early in the course of therapy; however, on continuous therapy with divided doses, up to 15% of the amount ingested daily could be demonstrated in active form in the urine. Results of studies on the mode of action of erythromycin and on the resistance of bacteria to that agent are presented in the succeeding papers.

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Resistance of Bacteria to Erythromycin.* (19816)

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In the course of studies on some of the antibiotic and other important biologic properties of erythromycin, a number of which were presented in a previous paper(1), observations were also made on the occurrence or development of resistance of bacteria to erythromycin. These included a search for resistant variants in cultures of "sensitive" strains, with and without exposures to the antibiotic. The development of resistance in bacteria during treatment of patients was also observed. Some of the details of these observations are presented here. The methods and materials were, for the most part, similar to those used in the preceding paper(1).

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† With the technical assistance of Clare Wilcox and Marilyn K. Broderick.

Search for Highly Resistant Variants in "Sensitive" Strains. Subcultures were made in broth from isolated colonies of the following organisms: *Streptococcus* C203, *Streptococcus* 98, a type 3 and a type 7 pneumococcus, *Staphylococcus* 193 and *Staphylococcus* 195. In the usual 2-fold broth-dilution tests the strains of streptococci and pneumococci were completely inhibited by 0.02 μg of erythromycin per ml and the staphylococcal strains by 0.2 μg per ml. The centrifugated sediment from 10 ml of each of these fully grown cultures was resuspended in a minimum volume of the supernatant fluid; a 2 mm loopful of each suspension was then streaked on the surface of a series of blood-agar plates, one of which contained no antibiotic while the others contained 2-fold dilutions of erythromycin from 800 to 100 μg per ml, inclusive. Each of these cultures grew luxuriantly on the antibiotic-free agar but no growth was discernible on any of the erythromycin-containing plates.

Variations in Resistance After Single Subcultures in Erythromycin. Two recently isolated strains of *Staphylococcus aureus* (No. 192 and 197) were used for this study; they were each completely inhibited by 0.4 μg of erythromycin per ml in the usual plate-dilution test. Isolated single colonies were picked from pour-plates of these cultures and they were subcultured in broth. When fully grown each was used for 2 further subcultures, one in the antibiotic-free broth and the other in broth containing 16 μg of erythromycin per ml, employing a 10% inoculum. After incubation for 48 hours, pour plates were made from these 4 cultures and 8 individual colonies were picked from each. These colonies, in turn, were subcultured in fresh broth and tested for sensitivity to erythromycin by the plate-dilution method.

The results are shown in Table I. The sensitivity of the cultures derived from the antibiotic-free media were all the same, within the limits of error of the method. The colonies derived from the growth in broth containing 16 μg of erythromycin per ml varied in their sensitivity over a range of concentrations from that which inhibited the parent strains to essentially that to which they had been exposed.

TABLE I. Sensitivity of Cultures of Single Colonies from Antibiotic-Free Broth and from Broth Containing Erythromycin.

	Min complete inhibiting conc., μg/ml	
	From antibiotic-free broth	
Colony	Staph. 192	Staph. 197
1	.4	.4
2		
3		
4		
5	.2	.4
6	.4	.8
7		
8		
	From broth containing erythromycin, 16 μg/ml	
1	.4	25
2	6.3	12.5
3	.4	.4
4	6.3	.4
5	12.5	12.5
6	12.5	.4
7	.2	12.5
8	6.3	12.5

Test done by plate-dilution method.

Effect of Repeated Subcultures in Graded Concentrations of Erythromycin. An attempt was made to develop strains with increased resistance to erythromycin by a method similar to that previously employed in this laboratory with other antibiotics(2, 3). Eight strains of "sensitive" bacteria, recently isolated from patients, were subcultured serially at 48-hour intervals on the surface of blood-agar plates containing 2-fold concentrations of erythromycin up to and including 100 μg per ml. The plates were freshly prepared at the time of each transfer and the inoculum for each subculture was a 2 mm loopful of a broth suspension of the growth from the surface of the plate containing the highest concentration of the drug on which the strain had previously shown good growth. The sensitivity of each strain at the time of each subculture is shown in Fig. 1.

The 4 strains of *Staphylococcus aureus,* one of which (No. 195) was subcultured serially on 2 occasions, all increased in resistance very rapidly so that they grew profusely on agar containing 100 μg/ml after only 3 to 5 subcultures on the erythromycin-containing medium. The strain of enterococcus also increased in resistance in about the same man-

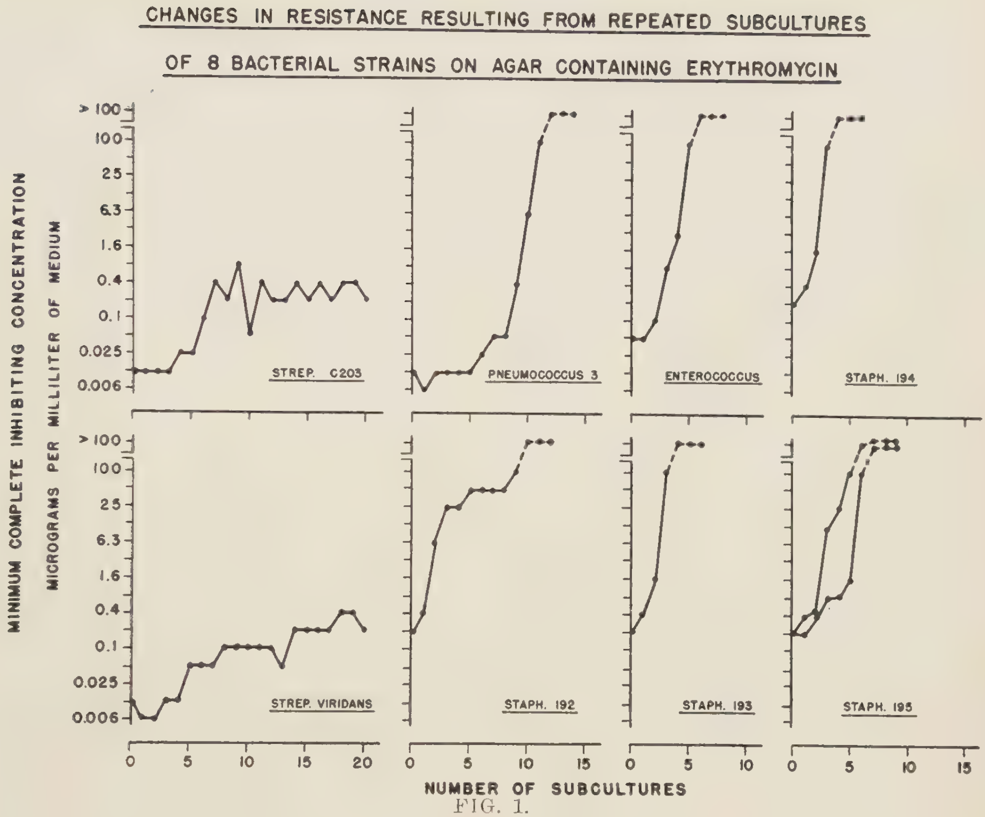

FIG. 1.

ner, while the type 3 pneumococcus reached the same level of resistance after 12 subcultures on erythromycin-agar. However, a group A hemolytic streptococcus and a strain of *Streptococcus viridans,* the latter from a patient with subacute bacterial endocarditis, increased only slightly in resistance after 20 transfers on the antibiotic-containing agar. The parent strains all retained their sensitivity unaltered throughout a parallel series of subcultures on similar plates without antibiotic. The results are summarized in Table II.

Cross-Resistance. The final subcultures of the 8 parent strains and of the 9 strains which had been transferred serially on erythromycin-agar were then all tested for sensitivity to penicillin, streptomycin, aureomycin, chloramphenicol, terramycin, bacitracin, polymyxin and neomycin. The results are shown in Table III. In almost every instance the strain which had been transferred without antibiotic and the corresponding one which had been repeatedly exposed to erythromycin were inhibited by the identical concentration of each of the other antibiotics. There were a few minor exceptions: the parent strains of type 3 pneumococcus and enterococcus appeared to be slightly more sensitive to penicillin than the corresponding erythromycin-resistant strains; the parent strain of *Staph.* 195 was inhibited by 1.6 μg/ml of terramycin and 25 μg/ml of chloramphenicol, while the erythromycin-derived strain of this organism was inhibited by 3.1 and 50 μg/ml of these antibiotics, respectively; and the type 3 pneumococcus which had not been exposed to erythromycin was inhibited by 400 μg/ml of neomycin while the corresponding erythromycin-resistant strain was inhibited by 100 μg/ml of neomycin. Except in the latter instances, the differences were only 2-fold (*i.e.,* one serial dilution) and were considered to be within the limits of error of the method; the sensitivity of the pneumococcus to neomycin, however, may have been significantly in-

TABLE II. Development of Erythromycin Resistance *In Vitro*.

Organism		Min complete inhibiting conc. of erythromycin (μg/ml)				
		Before subcultures	After serial subcultures on blood-agar		After 10 additional subcultures in antibiotic-free broth	
			Without antibiotic (Series P)	Containing erythromycin (Series R) †	Series P	Series R‡
Pneumococcus, type 3		.02	.04	>100 (13)	.02	400
Streptococcus C203		"	"	.4 (20)	"	.2
Streptococcus viridans		"	"	.4 (20)	"	.4
Enterococcus		.1	.4	>100 (9)	.2	>1600
Staphylococcus	192	.4	"	" (11)	.4	1600
	193	"	"	" (5)	.2	>1600
	194	"	"	" (5)	.4	
	195	"	"	" (9)	"	
	195(a)*	"	"	" (7)	"	

* A second series of subcultures with the same strain.

† The highest concentration of erythromycin used in this test was 100 μg/ml. The number of subcultures is given in parentheses and is the same for the corresponding strain in Series P.

‡ The highest concentration used in this test was 1600 μg/ml.

creased by the exposure to erythromycin.

Effect of Further Subcultures in Antibiotic-Free Broth. The final subcultures of each strain on the erythromycin-agar and the corresponding parent strains after they had been subcultured without antibiotic in the previous experiment were all subsequently transferred serially 10 additional times in antibiotic-free broth. They were then all tested again for sensitivity to erythromycin by the plate-dilution method. There was no significant change in the resistance of any of these strains as a result of the 10 additional subcultures. The "resistant" strains at this time were tested in concentrations up to 1600 μg/ml; the pneumococcus was found to be inhibited by 400 μg/ml, the enterococcus and 4 of the staphylococcal strains were only slightly inhibited by 1600 μg/ml, while *Staph.* 192 was completely inhibited by the latter concentration.

Biologic Effects of Exposures to Erythromycin. All the 8 strains which were included in the preceding study were tested for some of the principal biologic properties by which they are usually characterized; these tests were done before and at intervals during and after the serial subcultures both with and without erythromycin. The pneumococcus retained its ability to produce capsular swelling with type 3 antipneumococcus rabbit serum and its colonial characteristics and morphology also were unchanged. The enterococcus retained its colonial and microscopic morphology and remained heat-resistant after it had become erythromycin-resistant. The group A hemolytic streptococcus and the strain of *Streptococcus viridans* each retained its own colonial and microscopic morphology and its characteristic hemolytic property after the repeated subcultures on erythromycin-containing media. The strains of *Staphylococcus aureus* were all hemolytic and produced coagulase at the start of the experiment. However, as the resistance of these strains to erythromycin increased, their coagulase-producing activity first became poor and then was completely lost while the hemolytic property was retained throughout. Coagulase production was not regained by the subsequent serial transfers of the resistant staphylococci in antibiotic-free broth but it remained intact in the organisms which had been subcultured without erythromycin. The resistant staphylococci in this study also showed other biochemical changes in that they coagulated milk poorly and failed to ferment mannitol.

Development of Resistance During Therapy. In the preliminary clinical studies(4) there was an opportunity to observe strains of the same offending organism before erythromycin was given and again after several days of therapy with this antibiotic. In 2 patients with scarlet fever, hemolytic streptococci which were isolated from nasopharyngeal cultures after one week of treatment with erythromycin were as sensitive as the pretreatment strains in each instance. On

TABLE III. Effect of Repeated Subcultures on Erythromycin-Containing Agar on Sensitivity of Bacteria to 8 Other Antibiotics.

	Min complete inhibiting conc., μg/ml															
	Penicillin		Aureomycin		Terramycin		Chloramphenicol		Streptomycin		Neomycin		Bacitracin		Polymyxin B	
Organism	P*	R*	P	R	P	R	P	R	P	R	P	R	P	R	P	R
Pneumococcus, type 3	.04	.08	.4	.4	3.1	3.1	6.3	6.3	100	100	400	100	3.1	3.1	>400	>400
Streptococcus C203	"	.04	.8	.8	"	"	"	"	200	200	200	200	1.6	1.6		
Streptococcus viridans	.1	.1	"	"	"	"	"	"	100	100	"	"	3.1	3.1		
Enterococcus	6.3	12.5	6.3	6.3	12.5	12.5	50	50	"	"	"	"	6.3	6.3		
Staphylococcus 192 " 193 " 194	>400	>400	100	100	>400	>400	12.5	12.5	"	"	100	100	3.1	3.1	400	400
" 195	.8	.8	.8	.8	1.6	3.1	25	50	"	"	200	200	"	"	"	"
" 195(a)†	"	"	1.6	1.6	3.1	3.1	12.5	12.5	"	"	100	100	"	"	>400‡	>400‡

* Series P—Subcultured repeatedly on blood agar without antibiotic. Series R—Subcultured repeatedly on blood agar containing graded concentrations of erythromycin. The numbers of these subcultures are shown in Table II.

† Second series of subcultures of Staphylococcus 195.

‡ Partial inhibition in 400 μg/ml, the highest concentration tested.

the other hand, increases in resistance were observed in strains of *Staphylococcus aureus* isolated after unsuccessful erythromycin treatment in 2 patients with acute bacterial endocarditis. In one of these patients the staphylococcus obtained from the blood culture before erythromycin therapy was started was completely inhibited by 0.4 μg/ml of that antibiotic while a strain of the same organism isolated from a blood culture made on the tenth day of erythromycin therapy and tested at the same time required 100 μg/ml for complete inhibition. In the second patient, strains of *Staphylococcus aureus* isolated on 4 occasions during the 8 weeks before erythromycin was started were each inhibited by 0.4 μg/ml while 2 strains isolated after 7 and 8 days of therapy, respectively, and tested at the same time as the others required 12.5 μg/ml of erythromycin for complete inhibition. Interestingly enough the resistant strains of staphylococci which were obtained from the blood of these 2 patients retained all of the biologic characteristics (including coagulase production) possessed by the sensitive organisms which were isolated before erythromycin therapy was started.

Summary and Conclusions. 1. Variants with high degrees of resistance to erythromycin were not isolated from small volumes of cultures of erythromycin-sensitive bacteria which had not previously been exposed to that antibiotic. 2. When cultures of erythromycin-sensitive staphylococci (which were completely inhibited by 0.4 μg/ml in the plate-dilution test) were grown from a 10% inoculum in broth in the presence of 16 μg/ml of erythromycin, strains of varying resistance could be recovered after 48 hours; these required concentrations of erythromycin ranging from 0.2 to 25 μg/ml for complete inhibition. 3. By repeated subcultures of erythromycin-sensitive bacteria on blood agar containing graded concentrations of erythromycin it was possible to obtain strains of increased resistance to this antibiotic. The rate at which this resistance increased varied markedly with the strain. Several strains of *Staphylococcus aureus,* an enterococcus and a pneumococcus each increased more than 512-fold in from 3 to 12 such subcultures. Strains of only moderately increased resistance were obtained after 20 similar subcultures of a strain of hemolytic streptococcus and one of *Streptococcus viridans.* 4. The strains of increased resistance resulting from repeated subcultures on the erythromycin-containing agar retained that resistance after 10 further subcultures in antibiotic-free broth. 5. Strains of increased resistance to erythromycin derived by repeated subcultures in that antibiotic were almost invariably similar to their respective parent strains in their sensitivity to 8 other antibiotics; however, an erythromycin-resistant strain of type 3 pneumococcus had apparently increased in sensitivity to neomycin following its repeated subcultures in erythromycin. 6. Staphylococcal strains of markedly increased resistance to erythromycin were obtained from blood cultures of 2 patients with endocarditis following 7-10 days of treatment with this antibiotic. 7. The colonial, morphologic and biochemical characteristics of the erythromycin-resistant strains obtained by repeated subcultures in erythromycin-containing media resembled those of the respective parent strains from which they were derived in almost every instance. The strains of *Staphylococcus aureus,* however, lost their ability to produce coagulase and exhibited other changes in their biochemical properties following their repeated subcultures in the presence of erythromycin. Similar changes were not observed in the 2 strains of *Staphylococcus aureus* which had apparently become resistant during erythromycin therapy.

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Observations on Mode of Action of Erythromycin.* (19817)

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The results of studies carried out in this laboratory on some of the important properties of erythromycin as an antibiotic agent and on the occurrence and development of resistance of bacteria to that agent were presented in the preceding communications(1,2). The original description of the isolation of erythromycin and of its physical, chemical and antimicrobial properties were reported by McGuire *et al.*(3) and additional clinical and laboratory observations were presented by Heilman *et al.*(4). Preliminary results of clinical trials of this agent, chiefly in pneumococcal, streptococcal and staphylococcal infections have also been recorded(3-5). Observations on the mode of action of erythromycin as determined by growth curves of organisms exposed to that agent are described in this paper.

Materials and Methods. The organisms used were: a group A hemolytic streptococcus, No. 98, generally employed in assays of penicillin; a recently isolated strain of a coagulase-positive, hemolytic *Staphylococcus aureus* (No. 194); and the MacLeod strain of *Escherichia coli.* A single, well segregated colony from an agar pour plate of each of these strains was picked into broth and subcultures of these colonies were used for the present studies. The media used were brain-heart infusion broth (Difco) and heart-infusion agar (Difco) each at pH 7.4±. Defibrinated horse blood, 0.5% in broth and 10% in agar, was added for enrichment and as an indicator. Erythromycin in crystalline

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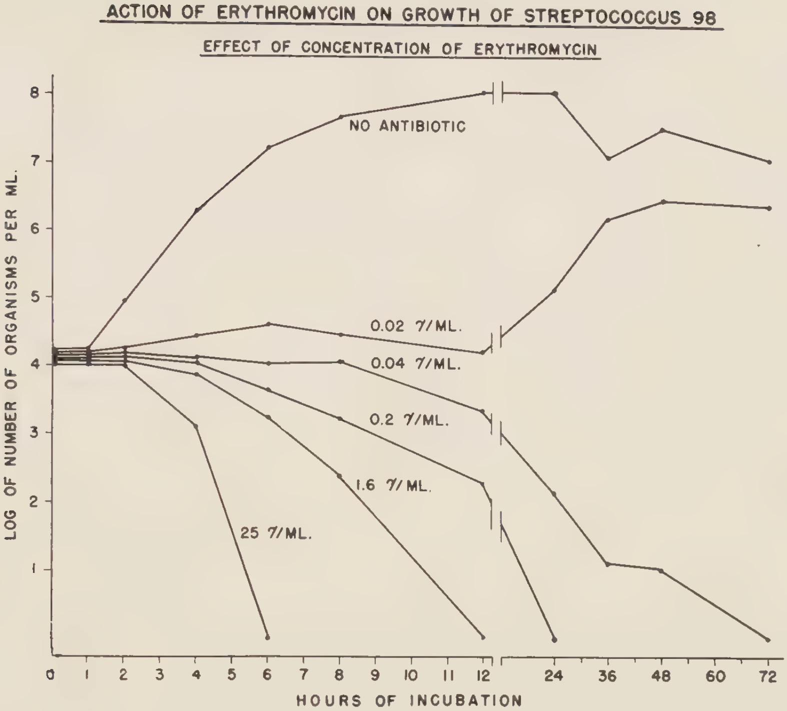
ACTION OF ERYTHROMYCIN ON GROWTH OF STREPTOCOCCUS 98

EFFECT OF CONCENTRATION OF ERYTHROMYCIN

FIG. 1.

form (890 μg of active base per mg) was furnished by Dr. J. W. Smith of Lilly Research Laboratories. Solutions of the antibiotic, usually 2000 μg of active base per ml, were made first in distilled water, using a few drops of ethanol to facilitate solution. Further dilutions of antibiotic and all those of the cultures were made in broth. Growth curves were made by starting with 10 ml of culture containing the desired inoculum of organisms and concentration of erythromycin and then making pour plate counts with 0.1 ml of the culture removed at appropriate intervals.

Growth in Varying Concentrations of Erythromycin. A series of 6 cultures of *Strep.* 98 were made in broth using the same inoculum (0.01% of a fully grown culture) in every instance; one of these contained no antibiotic while the others contained graded concentrations of erythromycin from 0.02 to 25 μg per ml. Pour-plate counts were then made at appropriate intervals during 72 hours of incubation at 37°C. The resulting growth curves are shown in Fig. 1. It is seen that with increasing concentrations of erythromycin there was roughly a proportionate decrease in the period during which the numbers of viable organisms remained stationary; after this period of bacteriostasis, the cultures containing 0.04 μg or more of erythromycin per ml all showed a steady decline in the number of viable organisms, the rate of this decline being roughly proportional to the concentration of the antibiotic. The time required to render these cultures entirely free of viable organisms was, therefore, inversely proportional to the concentration of erythromycin, within the range of concentrations employed. In 0.02 μg/ml, a 24 hour period of bacteriostasis (during which there was essentially a stationary number of viable organisms) was followed by multiplication over a period of another 24 hours during which the number of viable organisms almost reached that of the control culture grown without antibiotic. Qualitatively similar growth curves were obtained in the same manner with *Staph.* 194, as shown in Fig. 2. Greater concentrations of erythromycin, however, were required to achieve effects comparable to those obtained with *Strep.* 98. With this staphylococcus, there was only a small additional effect

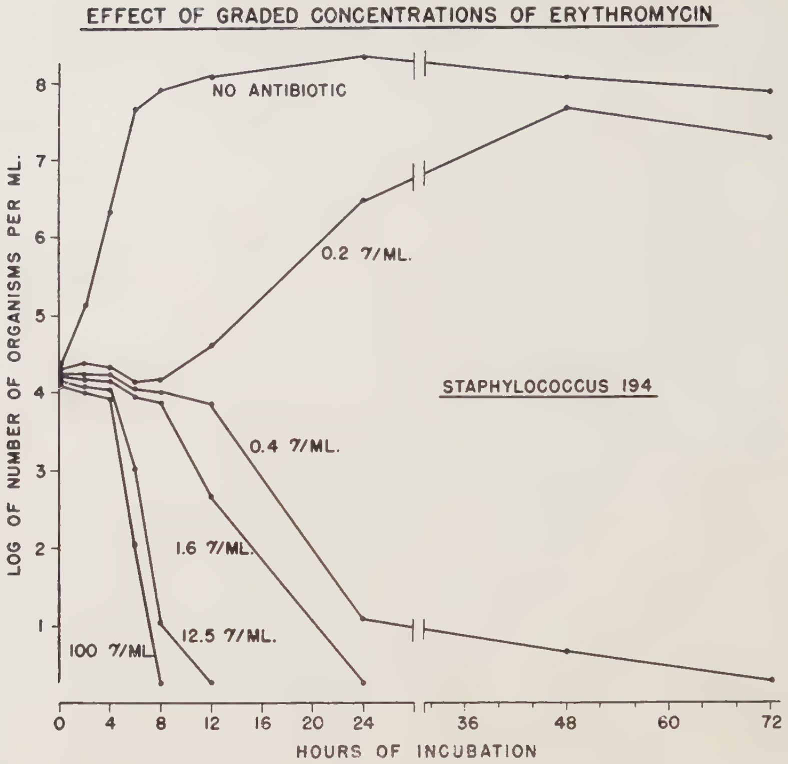

FIG. 2.

from 100 μg per ml of erythromycin over that exerted on the growth of this organism by a concentration of 12.5 μg per ml.

Growth from Inocula of Varying Size. A series of 8 broth cultures were seeded in pairs with 100-fold dilutions of a fully grown culture of *Strep.* 98, beginning with a 1% inoculum. One culture of each pair contained erythromycin in a concentration of 1.6 μg per ml while the other served as a control of growth without antibiotic. The resulting growth curves are shown in Fig. 3. The control cultures all grew in normal fashion and achieved the same maximum numbers in about 12 hours. The cultures grown in erythromycin, 1.6 μg per ml all showed a similar period of bacteriostasis, namely about 2 hours, after which the numbers of organisms began to decline at about the same rate in each of the cultures. The time required until no viable organisms could be demonstrated was, therefore, roughly proportional to the number of organisms per ml of the starting culture.

Incubation of Fully Grown Culture with Erythromycin. In this experiment a culture of *Strep.* 98 was grown for 24 hours after which 10 ml aliquots were measured into 4 culture tubes. Erythromycin in a volume of 0.1 ml of broth was then added to 3 of the tubes so as to make final concentrations of 0.4, 1.6 and 6.3 μg per ml, respectively, and 0.1 ml of antibiotic-free broth was added to the fourth tube. All 4 tubes were then incubated at 37°C and pour plate counts were made from each after various intervals. The results are shown in Figure 4. There was no significant difference in the numbers of viable organisms found in each of the 4 cultures throughout the period of observation.

Effect of the Phase of Growth. A series of 8 tubes of broth were inoculated at the same time and in an identical manner so that each contained a 10^{-6} dilution of a fully grown culture of *Strep.* 98 (approximately 100 colonies per ml in pour plate counts). One of these cultures served as a control, while to each of the others, in succession erythromycin

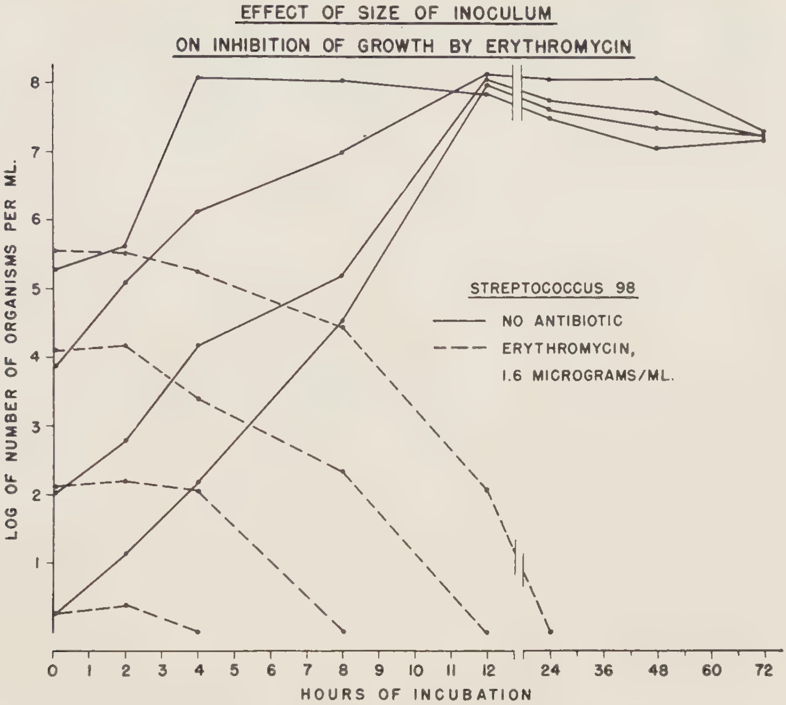

FIG. 3.

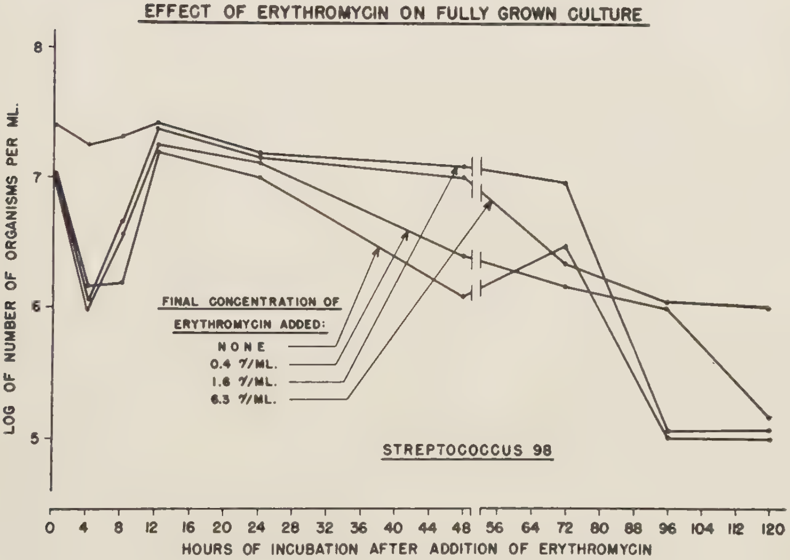

FIG. 4.

(in 0.1 ml of broth) was added after various periods of incubation up to 48 hours. The final concentration of erythromycin in each of the 7 cultures was 1.6 μg per ml. Pour plate counts were made from each of these cultures before and at intervals after the erythromycin was added and from the control culture throughout the experiment. The resulting series of growth curves is shown in Fig. 5. As might be expected from the previous ex-

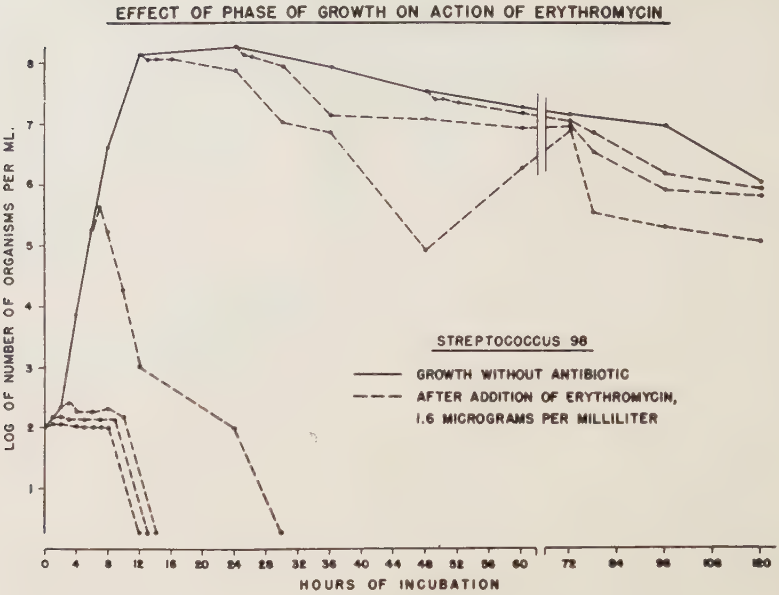

FIG. 5.

periments, as shown in Figures 3 and 4, when the antibiotic was added at 0, 1 or 2 hours and the numbers of organisms at the time of this addition was about the same, the resulting growth curves were very similar. When the erythromycin was added during the most active phase of growth, namely after 6 hours, there was a brief period of bacteriostasis, after which the number of organisms declined at a rapid rate. The culture that had just achieved full growth when the erythromycin was added showed a significant decline in the number of viable organisms at first with some subsequent growth. The addition of erythromycin after the stationary phase of growth had already been attained for several hours resulted in no significant decrease in the number of viable organisms as compared with the control culture to which antibiotic was not added.

Effect of Erythromycin at 4°C. Two series of broth cultures of *Strep.* 98, one undiluted and the other diluted 1:1000 from a fully grown culture, were kept in a refrigerator at approximately 4°C. One culture of each series contained no antibiotic while graded concentrations of erythromycin were added to the others at the time they were stored. Pour plate counts were then made of each culture at intervals up to 5 days. The exposure to erythromycin in the concentrations used at this temperature had no significant effect on the numbers of viable organisms found at each interval, in either series, as compared with those found in the corresponding cultures in the antibiotic-free broth.

Action of Erythromycin on Coliform Organisms. Cultures of *E. coli,* MacLeod, using 0.01% inoculum from a fully grown culture, were made in antibiotic-free broth and in broth containing erythromycin concentrations ranging from 1.6–400 μg/ml. Growth curves of these cultures indicated that in the lower concentration of erythromycin there was a period of bacteriostasis with some decline in the number of viable organisms and this was followed by multiplication until the numbers approximated those of the control group without antibiotics. In concentrations of 25–400 μg/ml of erythromycin there was a slow and steady depletion of the cultures but viable organisms were still demonstrated after long periods of incubation. Further studies with these organisms are in progress.

Conclusions. Erythromycin may be either bacteriostatic or bactericidal depending on the sensitivity of the organism and on the concentration of the antibiotic. Erythromycin exerts its effect only against multiplying bacteria.

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Distribution of Amethopterin in Normal Mouse Tissues Following Intravenous Injection.* (19818)

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The antifolic activity of amethopterin (4-amino-N^{10}-methyl pteroyl-glutamic acid) has been well established, bacteriologically(1) and by animal experiments(2-3). It has been shown to be effective in prolonging the survival time of leukemic mice(4) presumably by an induced relative deficiency of pteroylglutamic acid (PGA) and citrovorum factor (CF) which retards the development of the leukemic process(5). In a similar manner it causes remissions in a significant percentage of acute leukemias in childhood(6-9).

Probably as a result of the lack of a satisfactory simple method for assaying amethopterin directly, little has been reported on the fate of this compound following its administration either to human subjects or to the experimental animal. Swendseid(10) using a back titration modification of the standard method for microbiological assay of PGA, has reported on the excretion of aminopterin (4-amino-pteroylglutamic acid) during treatment of leukemic patients. The recent development(11) of a simple disc method for determining the concentration of amethopterin in blood and urine led us to broaden the scope of this technic to assist in the study of the metabolism of this compound in the normal mouse. As a preliminary to these studies, the distribution of amethopterin in the tissues of the normal mouse following intravenous injection was determined.

Materials and Method. Young mice of the inbred Akm stock, weighing approximately 20 g each, were used. All the mice were maintained on a standard diet of Purina Laboratory Chow prior to and during the experiment. Water was allowed *ad libitum* throughout the experiment. Each mouse was given a single injection of amethopterin at a dose of 0.5 mg/kg by the intravenous route. This was considered an optimum dose, producing readily assayable amounts in the tissues and yet it was well below the maximum tolerated daily dose, and approximately 1/200th of the acute LD_{50} dose(2). Mice were sacrificed at 15, 30, 60, 120, 180, and 240 minutes after injection. Under ether anesthesia, blood was collected in a heparinized syringe by severing the axillary vessels, the animal being finally killed by decapitation. In those mice injected 1, 2, and 4 hours previously the liver, spleen, kidneys and lungs were then removed

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‡ Research Fellow of the National Cancer Institute.

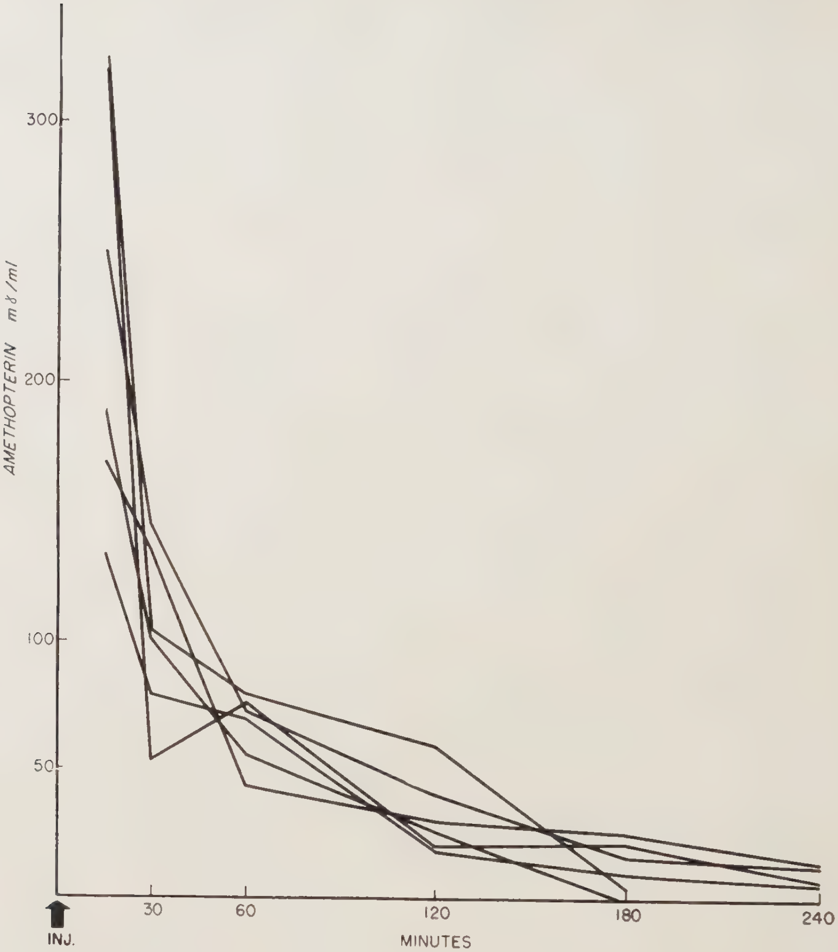

FIG. 1. Serum levels of amethopterin in normal mice after i.v. injection at .5 mg/kg.

and a sample of muscle taken from the hind leg. Each specimen was weighed and homogenized in distilled water immediately after removal.

Assay of Homogenate for Amethopterin Content. The samples were divided into 2 groups: (A) Unboiled, and (B) Homogenate boiled immediately for 5 minutes to destroy autolytic enzymes. The samples from group (B) were centrifuged and the supernatant assayed. In Group (A) the whole homogenate was assayed. Suitable dilution was carried out in both groups, following which 0.09 ml was delivered on to paper discs (Schleicher & Schuell penicillin assay discs, 12.7 mm diameter) placed on a pourplate of Difco folic acid assay medium containing 1 mγ/ml of PGA inoculated with *Streptococcus faecalis* (ATCC 8043). Following incubation over night the zones of inhibition were measured and calculated against standard zones of known concentration of amethopterin(11).

Results. Serum Levels. Fig. 1 shows the levels of amethopterin in the serum in 6 series of mice. It will be seen that after the first 30 minutes following injection the levels are closely related. In all cases the serum concentration has either reached or approximated zero in 4 hours.

Tissue Concentration. Table I shows the concentration of amethopterin in the tissues. It will be observed that much higher concentrations of amethopterin were assayable in the boiled specimens (B) in the case of the liver

TABLE I. Average Concentration of Amethopterin in Tissues of the Mouse Following i.v. Injection at .5 mg/kg Expressed in $m\gamma$.

Time following inj.	1 hr			2 hr			4 hr			24 hr	
	A	B		A	B		A	B		B	
		Content, $m\gamma$	$m\gamma$/g		Content, $m\gamma$	$m\gamma$/g		Content, $m\gamma$	$m\gamma$/g	Content, $m\gamma$	$m\gamma$/g
Liver (1–1.3 g)	424(6)	1400(6)	1215	0(3) 369(3)	868(6)	734	0(6)	628(6)	594	449(4)	410
Kidneys (.25–.35 g)	0(4)	227(6)	712	0(4)	162(6)	562	0(4)	120(6)	412	127(4)	384
Spleen (.07–.1 g)	0(3) 33(1)	20(6)	225	0(3) 22(1)	26(6)	273	0(3) 25(1)	20(6)	200	20(1) 0(3)	210 0
Lung (.15–.2 g)	0(3) 90(1)	0(3) 16(3)	0 96	0(4)	0(5) 15(1)	0 72	0(4)	0(5) 18(1)	0 84	0(4)	0
Muscle (.2–.25 g)	—	0(3) 5(1)	0 25	—	0(4)	0	—	0(3) 10(1)	0 40	0(4)	0

A—Unboiled specimens ($m\gamma$/organ). B—Boiled specimens (total content in $m\gamma$/organ and conc. in $m\gamma$/g).

Figures in parentheses denote No. of mice tested.

and kidneys and to a lesser extent in the spleen. This was readily explained by the fact that the enzymatic freeing of folic acid and CF from their conjugates was prevented by boiling. In the unboiled specimens (A) on the other hand, incubation resulted in freeing of folic acid and CF and hence reversal of some or all of the amethopterin activity. This was significant as it was an indication as to the folic acid and CF content of the tissue and suggested that the highest concentration was present in the liver and kidneys. Owing to the small quantity of free folic acid and CF present in tissues(12) it was presumed that the boiled specimens gave an accurate estimate of the amount of amethopterin present in the tissues. It can therefore be observed that the majority of the compound was present in the liver, followed in sequence by the kidney and spleen, while no significant quantities were found to be present in the lung after one hour and at no time in the muscle.

The retention of relatively large quantities of amethopterin in the liver and kidneys after 4 hours, at which time it had approximated zero in the serum, prompted us to determine the amount of the compound present after 24 hours. As may be seen, readily assayable quantities, little different from those found after 4 hours, remained.

Discussion. It was shown by this experiment that following the administration of amethopterin, it became concentrated in folic acid and CF-containing tissues. In addition it was observed that the highest concentration of amethopterin was found to be present in the liver followed in order by the kidney and spleen. This is of interest in view of the fact that a similar distribution of folic acid and CF has been demonstrated in the rat, guinea pig and chick tissues by direct microbiological assay(12). This tissue selectivity of amethopterin is not unexpected in view of the known properties of this compound, and supports the view that the antimetabolite replaces the metabolite (in this case amethopterin, and folic acid and CF) in the enzyme systems in which it is associated. A similar experiment has since been carried out in mice with advanced leukemia (Ak4). The results to date suggest a more widespread distribution and higher concentration of amethopterin in the tissues, which may be an indication of the affinity of the leukemic cell for amethopterin. In view of a correspondingly high serum level, however, the possibility of these findings being a consequence of renal dysfunction secondary to the leukemic process cannot be excluded.

Another finding of interest was the presence of amethopterin in significant quantities (approximately 6% of the injected dose) in the liver and kidney 24 hours after injection. Its presence was even more interesting since the serum level had almost reached zero within 4 hours of administration.

Previous studies(11) have shown that following oral or intramuscular administration to normal adult human subjects of amethopterin at a dose of 5 mg, 40-57% was excreted in 24 hours, the majority within 4-6 hours. Applying the knowledge obtained from the above experiment (Table I) it appears safe to presume that the remainder of the drug is metabolized or retained in the tissues. It is suggested from this experiment that following an injection of amethopterin most of the compound is rapidly excreted or metabolized within 4 hours, the remainder being concentrated in folic acid and CF-containing tissues. The continued presence of amethopterin activity in these tissues after 24 hours or more indicates a failure to complete the metabolism of amethopterin.

It will be noticed that we have referred to the substance in the tissues with growth-inhibiting properties for *Streptococcus faecalis* as amethopterin. Preliminary chromatographic studies suggest that this substance may indeed be amethopterin. However, the possibility that it may be the deaminated derivative, N^{10}-methyl-PGA, cannot be excluded at present as both have identical Rf values in the aqueous Butanol and acetic acid system and both are inhibitory to *S. faecalis*. In an attempt to reach a more definite conclusion as to its nature, however, further chromatographic studies are in progress.

Summary. 1. The concentration of amethopterin in the serum, liver, kidney, spleen, lungs and muscle of normal mice at intervals following intravenous injection of 0.5 mg/kg has been ascertained. 2. The relative concentrations of amethopterin in the tissue appeared to parallel the folic acid and CF content *viz.* liver, kidney, and spleen in descending order. No significant quantity of amethopterin was found in the lungs or muscle. 3. Amethopterin-like activity was found to be still present in the liver and kidney in relatively large quantities 24 hours after injection although the serum level had reached or approximated zero in 4 hours.

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In vivo Reversal by Cysteine of the Virustatic Effect of a Nitrofuran.* (19819)

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Previous observations in this laboratory have shown that 5-nitro-2-furaldehyde semicarbazones, or semioxamazones inhibit viruses of the psittacosis-lymphogranuloma group and the agent of primary atypical pneumonia in chick embryos and experimental animals (1,2). Recently Green and his collaborators have found the 5-nitro-2-furaldehyde semicarbazone (Furacin) acts as a limited sulfhydryl inhibitor reversible by cysteine, and that bacteria made resistant to Furacin show cross resistance to p-chloromercuribenzoate and trivalent arsenicals(3). Modifications to resistance appeared to be associated with changes in the SH groups of certain of the bacterial enzymes(4). Reduction of Furacin by tissues(5), bacteria, or cell free extracts is probably linked to a DPN-flavoprotein system(6,7) and occurs in the presence of a wide variety of oxidizable substrates. Cysteine accelerates the destruction of Furacin anaerobically in the presence of a partially purified xanthine-oxidase system from milk(8). This paper will report the reversal by cysteine of the inhibition of meningopneumonitis virus in the allantoic sac and present some experiments designed to indicate the site of reversal.

Materials and methods. Meningopneumonitis virus strain Cal 10 as used in these experiments had been carried through more than 30 yolk sac passages and 9 passages in the allantoic sac. Titrations of the ID_{50} were done in the allantoic sac and LD_{50} in the yolk sac. The compound subsequently referred to as NF 185 is 5-nitro-2-furaldehyde 4(diethylaminopropyl) semicarbazone. This drug because of its greater solubility was used in preference to Furacin. Solutions were sterilized by filtration through an ultra-fine Corning sintered glass filter. Cysteine hydrochloride solutions were sterilized by heat and after neutralization with NaOH were kept at 4°C under a vaseline seal. The degree of infection of the chick embryos was estimated by subinoculation of undiluted allantoic fluids into mice by the intranasal route and measuring the degree of pulmonary consolidation in terms of percentile lesion scores as previously described(1). NF 185 in the allantoic fluid was estimated by a modification of the spectrophotometric method described by Paul, *et al.*(9).

Effect of NF 185 on meningopneumonitis virus in embryonated eggs. To test the effect of NF 185 on the growth of meningopneumonitis virus in the allantoic sac of the chick embryo the following experimental setup has been adopted. The virus was inoculated into the allantoic sac in the amount of 100 I.D. contained in a volume of 0.1 cc. In order to avoid an immediate direct contact with the virus, the drug was inoculated into the yolk sac in a volume of 0.5 cc of solution. Both inoculations were performed within an hour. The age of the embryos used varied between 10 and 12 days. After inoculation the eggs were incubated for 5 days at 35°C and at the end of this period allantoic fluid from each egg was tested in 2 mice.

The results of these experiments are recorded in Table I. While all the control eggs at the end of the incubation period appeared heavily infected most of the eggs that received the drug did not contain enough virus to produce lung lesions in mice. Those found to be infected seemed to contain a much smaller amount of virus as compared with the controls. The data show that NF 185 has a chemotherapeutic effect against meningopneumonitis virus similar to that found for other nitrofurans(1) and that the minimal effective dose is between 0.5 and 1.0 mg under the conditions of these experiments.

Effect of NF 185 on meningopneumonitis virus in vitro. To establish whether the action of NF 185 on meningopneumonitis virus was

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† World Health Organization Fellow.

TABLE I. Effect of NF 185 Injected into Yolk Sac on Growth of Meningopneumonitis Virus in Allantoic Sac.

Exp. No.	NF 185 in y.s., mg	No. of eggs containing detectable virus/total	Avg lesion score in mice†
1	2	*	
	1	1/5	2
	.5	3/4	24
	0 (controls)	5/5	76
2–5‡	1	4/16	5
	0 (controls)	21/21	87

* All the embryos died between the 1st and 3rd day after inoculation due to toxicity of the drug.

† Lesion score in %; avg for entire group of eggs. A lesion score of 100 represents death with complete pulmonary consolidation. In surviving mice 80 represents on the avg 4+ consolidation; 60, 3+; 40, 2+; and 20, 1+.

‡ Summary of results of 4 exp.

of virucidal or virustatic type, serial 10-fold dilutions of the virus were made in normal allantoic fluid and were incubated with NF 185 at concentrations of 200 γ/cc and 50 γ/cc including saline control for 6 hours at 35°C. At the end of the incubation 0.5 cc of the mixtures which originally contained 2,000, 200 and 20 LD_{50}/cc with each amount of drug were inoculated into the yolk sac of four 7-day-old embryos and the eggs incubated at 35°C for 12 days. The eggs were candled daily and the deaths recorded. There was no significant difference in mortality rate or time of death between the eggs inoculated with drug-virus mixtures and the controls with virus alone. The data demonstrate that NF 185, even in a concentration about 10 times that encountered in the allantoic fluid under our experimental conditions, does not seem to exert any appreciable virucidal effect *in vitro* on the meningopneumonitis virus.

Reversal by cysteine of the effect of NF 185 on virus growth. As an approach to the knowledge of the mechanism of action of NF 185 and also of chloramphenicol we tested two compounds, cysteine and p-hydroxybenzoic acid, as possible antagonists of antiviral activity. The inhibitors and virus were injected into the eggs as previously described. Cysteine or p-hydroxybenzoic acid was injected into the allantoic sac. In the experiments with cysteine this was given in daily doses of 10 mg and the treatment was started at different times after the inoculation of virus and nitrofuran. When the treatment with cysteine was begun at four days the eggs were incubated for 7 days instead of the usual time of 5 days. At the end of the incubation period the allantoic fluids were tested for presence of virus by intranasal inoculation in mice. The results in Table II clearly show that the injection of cysteine into the allantoic sac reverses the inhibition of virus growth caused by NF 185. It appears also that the reversal is possible as late as 96 hours after the inoculation of the virus and the drug. This finding suggests that the action of NF 185, as already observed in the experiments *in vitro*, is mainly of virustatic type. The virus is not killed by the nitrofuran but it remains in the allantoic sac for as long as four days without significant multiplication until cysteine is administered; then it promptly starts to increase. In eggs infected with meningopneumonitis virus and treated with minimal effective doses of 0.2 mg aureomycin or 1.0 mg of chloramphenicol, cysteine given as above did not have any effect on the inhibitory activity of these antibiotics.

Since the work of Davis(10) has shown that p-hydroxybenzoic acid is a growth factor for certain bacteria and that this substance reverses the bacteriostatic activity of p-nitrobenzoic acid, experiments similar to the above were undertaken both with NF 185 and with chloramphenicol. p-Hydroxybenzoic acid given either as a single dose of 10 mg or in daily doses of 5 mg for 5 days failed to diminish the virustatic activity of the 2 nitro compounds.

Evidence of the presence of NF 185 in the allantoic fluid after inoculation into the yolk sac. Eggs on the 10th day of incubation were inoculated into the yolk sac with 1 mg of NF 185 dissolved in 0.5 cc of saline. Another group of eggs of the same age was given 0.5 cc of saline only. The eggs were incubated at 35°C and at different intervals of time the allantoic fluids of 4 eggs of each group were harvested. If the allantoic fluid appeared grossly contaminated with blood it was discarded. The fluids were centrifuged for 15 minutes at 2,000 r.p.m., diluted 1:2 with saline and examined at the spectrophotometer

TABLE II. Reversal of Cysteine of Antiviral Effect of NF 185.

Cysteine, 10 mg daily, started at....hr	Eggs treated with		Allantoic fluid tested for presence of virus at....days	No. of eggs containing detectable virus/total	Avg lesion score in mice
	Y.S.	All.S.			
—	NF 185	S*	5	3/15	9
0	185	C	”	8/8	73
48	185	C	”	5/5	62
0	Saline	C	”	8/8	82
—	Saline	S	”	15/15	84
—	NF 185	S	7	3/11	9
72	185	C	”	5/5	82
96	185	C	”	9/9	70
—	Saline	S	”	12/12	81

* S = Saline; C = Cysteine.

Virus 100 ID_{50} in allantoic sac + NF 185 1 mg in yolk sac at time 0.

TABLE III. NF 185 in Allantoic Fluid at Different Intervals after Injection into Yolk Sac.

Hr after inj.	No. of eggs		Optical density of allantoic fluids at 380 mμ				Diff. avg	NF 185, γ/cc		
			NF 185		Saline					
	NF 185	Saline	Range	Avg	Range	Avg		Min	Max	Avg
4	3	3	80- 90	85	70- 90	80				
8	3	3	145- 200	171	75- 85	80	91	3	6.5	4
16	3	3	290- 340	318	85- 95	90	228	8.4	10.4	9.6
24	4	4	430- 920	670	85-135	105	565	15.6	34.4	24
38	4	4	460- 670	545	95-200	147	398	13	22	17
48	4	4	410- 820	610	95-220	190	420	10	26.4	18
72	4	4	450-1100	681	165-265	217	464	10	37	20.6

in the region from 300 to 420 mμ; readings were taken at intervals of 10 mμ. A standard solution of NF 185 in saline containing 20 γ/cc was run in parallel with the allantoic fluids.

The first significant variation in the spectral absorption curve of the allantoic fluid from eggs that received the nitrofuran, as compared with the allantoic fluid of the control eggs, was observed 8 hours after the inoculation. The variation consisted in an increase of the optical density with its maximum at 380 mμ, corresponding to the peak of the absorption curve of NF 185. On the basis of the spectrophotometric analysis no evidence has been found for the appearance of other products possibly derived from the metabolism of the nitrofuran. The concentration of nitrofuran in the allantoic fluid was estimated by subtracting the average optical density of the allantoic fluids of the control eggs from the optical density of the allantoic fluids of eggs that were injected with nitrofuran. The concentration of nitrofuran was then established by comparing the values obtained with a standard titration curve. The results of these measurements are given in Table III.

Effect of cysteine injected into the allantoic sac on the nitrofuran content of allantoic fluid. A group of eggs that had received 1 mg NF 185 into the yolk sac 24 hours previously was given 10 mg of cysteine into the allantoic sac. Another group of eggs that had received the same amount of NF at the same time was given saline as control. The eggs were incubated at 35°C and at intervals of 6, 12 and 24 hours after the injection of cysteine the allantoic fluids from 3 eggs were examined for their NF content as previously described. A nitro-prusside test for the –SH group was also performed on each fluid.

In Table IV are recorded the results of this experiment. It will be seen that cysteine causes a temporary disappearance of the nitrofuran from the allantoic sac. The level is very low after 6 hours but by 12 hours it has returned to about half of the control value and at 24 hours there is little difference be-

TABLE IV. Analysis of Allantoic Fluid for NF 185 after Addition of Cysteine.

Hr after inj. of cysteine	NF 185 γ/cc in allantoic fluid from eggs given		Nitroprusside test*
	Cysteine	Saline	
6	3.5	24.6	+
12	12.4	19	—
24	20.4	22.8	—

* Nitroprusside test neg on eggs receiving saline.

tween the cysteine-treated and control groups. Assuming a concentration of cysteine in the allantoic fluid immediately after addition of 1 mg per cc the molar ratio of cysteine to NF 185 is of the order of 100:1. After the rapid disappearance of the cysteine the nitrofuran tends to reaccumulate in the allantoic sac.

Discussion. Cysteine added in the test tube to allantoic fluid containing NF 185 produces only a small reduction (about 10%) of the nitro-compound. The observed decrease of nitrofuran in the allantoic sac must be due, therefore, either to the activity of the lining cells of the sac or to enzymes in the allantoic fluid. The temporary reduction of the concentration of inhibitor in the fluid may result in a decreased amount within the cells thus permitting growth of virus. Since the nitrofuran soon reappears in the allantoic fluid it seems unlikely that there are important changes in the blood concentration.

Because of the lack of *in vitro* action of the nitrofuran at the experimental concentrations on meningopneumonitis virus it can reasonably be assumed that the important effects are produced in the cells lining the allantoic sac. It is not possible to tell on the basis of these experiments whether the inhibition of growth is due to an action on the virus itself or to disturbance of the metabolic activities of the host. If the cysteine acts intracellularly the observed reversal of inhibition could come about through dissociation of the nitrofuran from a sulfhydryl complex or by an increased rate of reduction of the nitrofuran to an inactive form. The experimental data indicate that the drug persists for long periods of time in the embryo after a single injection into the yolk sac. Reversal of its effect by cysteine as late as 96 hours after infection supports the concept of a prolonged virustatic activity in the tissues of the allantoic sac.

Summary. 1. The virustatic effect of 5-nitro-2-furaldehyde 4(diethylaminopropyl) semicarbazone on meningopneumonitis virus in the allantoic sac is reversed by cysteine given as late as 96 hours after the drug and virus. Injection of 10 mg of cysteine reduces temporarily the amount of nitrofuran in the allantoic fluid. 2. Since the nitrofuran had no *in vitro* virucidal effect at the concentrations used, the observed virustatic effect and its reversal are probably intracellular. 3. Cysteine did not reverse the virustatic effect of chloramphenicol or aureomycin.

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Relaxation of Pubic Symphysis of the Mouse during the Estrous Cycle and Pseudopregnancy.* (19820)

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In the course of recent investigations on the role of the relaxative hormone in the mouse(1-3), a study was made of its possible significance during the estrous cycle and pseudopregnancy. Previously, Gardner(4) had found that the pubic symphysis of the male mouse remains cartilaginous but, in the female, the cartilage is replaced by an interpubic ligament at sexual maturity. This sexual dimorphism, due to the action of estrogen, could be prevented by castration. It has also been found that during pregnancy the pubic bones of the mouse begin to separate on about the 13th day and reach a maximum by the 19th day, the rate of separation being about one millimeter per day(5). Castration and destruction of the fetuses prevented this separation(6). In the castrated mouse, estrogen could not reproduce the separation seen in pregnancy while combinations of estrogen and an extract of pregnant rabbit serum (PRS) did. Therefore, Hall and Newton(5,7) concluded that pubic separation occurring during pregnancy is caused by relaxative hormone.

In this study, x-ray measurements of the separation of the pubic bones were made during various stages of the estrous cycle and during pseudopregnancy.

Materials and Methods. Three separate groups of mature, virgin mice were studied at different times (Table I). Vaginal smears were taken from each mouse, dried, fixed in methanol and stained in 0.05% Giemsa solution. The cellular contents of these smears were evaluated and each of the mice was recorded as being in estrus, metestrus, diestrus, or pro-estrus, according to the classification of Snell(8). Immediately before the vaginal smears were taken, x-ray photographs were taken of the pubic symphyses and the separations were measured by a technic already described(2). A total of 162 mice were used for this part of the study. Another group of 14 mice was made pseudopregnant by stimulation of the uterine cervix during estrus with a faradic current of five volts. Three days later, the uteri of 9 of these mice were traumatized with a thread to produce a decidual reaction. All mice were x-rayed several times during the course of pseudopregnancy and vaginal smears were taken daily to verify that they were pseudopregnant.

Results. During estrus, there is a small but definite relaxation of the pubic symphysis (Table I). In each of the 3 experiments, the average separation of the symphysis during estrus was larger than the average separation at any other time in the cycle, and when the total results were averaged it was found that the average separation of 0.43 mm in 70 estrous mice was approximately twice that which occurred in the other mice during diestrus, proestrus or metestrus. A statistical analysis of these data verified that the differences were highly significant with a P value of less than 0.005. Of the 14 mice stimulated during estrus and thereby made pseudopregnant, none demonstrated a significant change in the separation of the pubic symphysis. The change found after 12 days of pseudopregnancy was 0.04 ± 0.03 mm (mean ± standard error) for the control mice indicating that pseudopregnancy did not effect a separation of the pubes. Similar measurements for the mice whose uteri had been traumatized showed a change of 0.01 ± 0.02 mm. Therefore, traumatization of the uterus, with the resultant production of deciduomata, also had no effect on the separation of the pubic bones.

Discussion. The pubic symphysis of the normal mouse shows an increased separation during estrus. The magnitude of this separation is such that it cannot be due to the presence of estrogen alone. For example,

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TABLE I. Separation of the Pubic Symphysis of the Mouse During Various Stages of the Estrus Cycle.

		Metestrus		Diestrus		Proestrus		Estrus	
Group	No.	No.	Avg separation (mm)	No.	Avg separation (mm)	No.	Avg separation (mm)	No.	Avg separation (mm)
I	92	11	.17	24	.20	13	.22	44	.40
II	50	5	.14	20	.21	8	.16	17	.35
III	20	2	.60	5	.08	4	.30	9	.69
Totals	162	18	.22	49	.19	25	.21	70	.43

Statistical analysis of averages of total number of animals in each group by "Student's" t test verified that differences between average of the estrous group and each of the other groups were highly significant (P values less than 0.005). Standard error for the estrous group is ±0.05 and for the other groups less than ±0.01 in each case.

after 8 to 10 days pretreatment with 1 μg estradiol daily, the separation in castrated mice is found to average only 0.03 mm per day for the last 2 days(2). In contrast to this, the increment for estrous mice is 0.22 mm for the day following proestrus (Table I). In castrated mice, after 2 days of estrogen priming an extract of PRS was able to produce an increase in separation of 0.31 mm in the next 24 hours(9). It is of interest that the pregnant mouse may show a rate of separation of 1.0 mm per day(5), and this rate can be duplicated experimentally only by the administration of estrogen and PRS extracts(2,7). Thus it appears that the phenomenon of relaxation during estrus as well as during pregnancy is caused by the combined action of estrogen and a relaxative hormone.

The fact that relaxation does not occur in the pseudopregnant mouse even in the presence of deciduomata does not conclusively verify the absence of a hormone of relaxation. Hall and Newton(5) have shown that even the pregnant mouse does not relax during the first 13 days of pregnancy. They have also found(10), and we have confirmed that progesterone prevents the occurrence of pelvic relaxation in mice treated with estrogen and extracts of PRS.

Summary. X-ray photographs were taken of the pubic symphyses of mice at various stages of the estrous cycle and the separations of the pubic bones were measured. Significant relaxation occurred during estrus. No change in separation could be detected during artificially induced pseudopregnancy even when the uteri were stimulated to produce deciduomata.

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Metabolic Effect of Serotonin in the Rat.* (19821)

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In the course of a study of some of the pharmacological properties of serotonin (5-hydroxytryptamine), we were led to observe the effect of this substance on the oxygen consumption of the rat. Marked depression of oxygen consumption lasting for periods of more than an hour was observed. This effect is interesting since it is opposite to what would be predicted for a compound structurally related to the sympathomimetic amines.

Materials and Methods. Mature albino rats of the Wistar strain weighing 160 to 220 g and of both sexes were employed. The apparatus was the closed circuit type, in which calcium chloride and soda lime were used to absorb water and carbon dioxide, respectively. Measurement of oxygen consumption was made at ten minute intervals, adding oxygen from a reservoir by displacement with saturated sodium chloride solution. The solution was run in from a burette until the equality of pressure inside and outside the system was reestablished. The serotonin employed was the synthetic creatinine sulfate monohydrate complex, but all results are presented in terms of the free base. Injections of the aqueous solution were made intraperitoneally in a volume of 0.5 cc or less.

Results and Discussion. The observations are summarized in the table which gives the weight, sex, and preinjection oxygen consumption (in grams per hour per 100 g of rat, as the mean of 3 to 6 ten minute periods) for each rat, as well as the temperature and dose level of each experiment. The effect on the oxygen consumption is presented in terms of the maximum effect observed for any ten minute period and the time in minutes for the oxygen consumption to return from the initially observed effect to 50% of the maximal one. In these studies an effect of less than 20% or lasting less than 20 minutes is not considered significant. It can be seen that the minimally active dose is 0.5 to 1.0 mg/kg, since 1 of 3 rats at 0.5 mg/kg while 3 of 4 rats at 1.0 mg/kg gave positive responses. With higher doses, the effect is more pronounced, especially with regard to duration; a maximum intensity of depression of oxygen consumption is reached at 2 to 4 mg/kg. A representative experiment, that of rat No. 8 in the table, is shown in the figure.

TABLE I. Depression of Oxygen Consumption by Intraperitoneal Injection of Serotonin in the Albino Rat.

Rat No.	Wt, g	Sex	Temp., °C	O_2 consump./hr/100 g rat, g	Dose, mg/kg	Max. change, %	Duration to 50% recovery, min.
1	200	F	24	.22	.5	—25	54
2	165	F	23	.21	.5	none	
3	200	M	28	.16	.5	—16	14
4	165	F	23	.24	1	—45	40
5	220	M	28	.19	1	—27	60
6	175	F	26	.14	1	—24	12*
7	210	F	28	.19	1	—29	30
8	205	F	24	.23	2	—53	55
9	160	F	25	.20	2	—30	22
10	200	M	23	.20	2	—48	49
11	215	M	23	.24	2	—48	57
12	165	F	24	.21	4	—40	50
13	185	F	28	.17	4	—44	70†
14	210	M	28	.23	4	—41	50
15	170	F	27	.23	4	—56	90
16	180	F	30	.16	4	+32	65
17	180	F	28	.23	4	—40	>120
18	195	F	28	.17	8	—60	65
19	220	F	29	.22	8	—45	>120‡
20	160	F	25	.23	8	—61	90

* 24 hr fasted. Preceded by rise to +39%.
† 48 hr fasted.
‡ Subcutaneous injection. Marked cyanosis.

These dose levels are far removed from those which show acute toxicity, the LD_{50} for the rat being about 50 mg/kg by intraperitoneal route(1), and 117 mg/kg subcutaneously(2), (see rat No. 19). The principal toxic symptom displayed was one of sporadic, spasmodic crawling movements during which the

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* This work was carried out under the auspices of a Fulbright research grant awarded to one of us (M.M.R.).

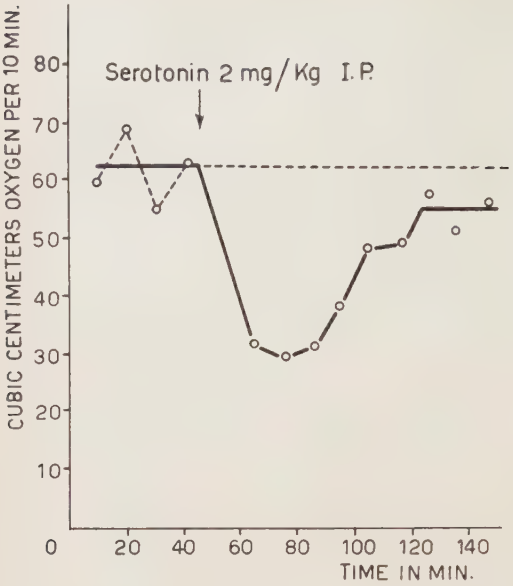

FIG. 1. Rat No. 8, female, 205 g.

animal appeared very disturbed. In general, respiratory frequency was somewhat depressed, changing from about 70 to 55 respirations per minute, but the decreased oxygen consumption has been observed also where the respiratory frequency was unchanged or even somewhat increased. About half of the rats displayed signs of irritation manifested by intense biting and scratching especially of the areas in the posterior regions. When this irritation was sufficiently pronounced, it could shorten the period of depressed oxygen consumption. This was the case with rat No. 9 in which the symptoms of irritation were greatest, and in which the period of metabolic depression was followed by one of increased oxygen consumption attaining a level of +38%.

The decrease in oxygen consumption is not necessarily related to the gross toxic reactions since two of the rats (Nos. 7 and 14) gave appreciable responses with very few toxic manifestations, while rat No. 16, the only animal which responded solely with an *increase* in the rate of oxygen consumption, showed the usual toxic symptoms. Rat No. 21, in which the temperature was measured before injection and during the period of maximal decrease in metabolic rate, showed a temperature drop from 37.4° to 33.2°.

The question naturally arises as to whether this effect represents a regulatory (hormonal) activity of serotonin or is rather a resultant of the pharmacological actions in the rat. The evidence in favor of the hormonal activity seems to rest with the wide distribution of the substance in both vertebrate and invertebrate species(3), and the fact that the activity is seen at a level of intraperitoneal injection as low as 1 mg/kg for a substance known to be rapidly inactivated by monoamine oxidase(2). The evidence in favor of the effect being pharmacological is somewhat stronger, in that no significant effect on oxygen consumption was seen in three other mammalian species: guinea pig, 20 mg/kg I.P.; rabbit, 10 mg/kg I.V., 4 and 50 mg/kg I.P.; dog (under chloralose anesthesia), 2 mg/kg I.V., 10 mg/kg I.P. This species difference may be related to the low monoamine oxidase content of the rat kidney.

Although serotonin is known to have several peripheral pharmacological actions which might contribute to reduced oxygen consumption, it may be that the effect being observed here is in large part due to an action on the central nervous system or on the arterial vessels of the brain, and that the rat is peculiarly susceptible to this action. Rat No. 18 showed a temporary fore and hind leg paralysis as well as narcotic effects, the former lasting an hour, the latter two finally disappearing overnight. Two other observations implicating such a site of action are: 1) the rat shows a marked increase in acute toxicity to serotonin under anesthesia(4), and 2) the pial arteries of the rat have been shown to be sensitive to the serum vasoconstrictor(5).

A similar depression of the rate of oxygen consumption was observed with both tryptamine and histamine when injected intraperitoneally in the rat. The dosages required to obtain an effect with these two amines, both of which are closely related structurally to 5-hydroxytryptamine, were 10 times greater than with the latter, and the effect itself was different with respect both to the immediacy of onset and the duration. With tryptamine the lowered rate of oxygen consumption appeared only after a period of 20 to 25 minutes following injection as compared with the immediate effect usually observed with serotonin. The period preceding an observable effect after histamine injection was even longer, and the duration of the effect was two hours or more. The toxic symptoms following administration of tryptamine were similar to those observed with serotonin; no toxic symptoms were seen following histamine injection.

Summary. Serotonin (5-hydroxytryptamine) has been found to depress the rate of oxygen consumption of the albino rat when injected intraperitoneally at dose levels as low as 1 mg/kg. A similar effect was observed with tryptamine and histamine (both of which are structurally related to serotonin) at much higher levels. This effect was not observed in the rabbit, guinea pig, or dog.

We wish to thank Professor Daniel Bovet for his advice and encouragement, and the Upjohn Company and Abbott Laboratories for their generous supply of serotonin creatinine sulfate.*

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Effect of Certain Carbohydrates on Nutritional Requirements of *Lactobacillus pentosus.* (19822)

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Lactobacillus pentosus, Strain 124-2, ferments both hexoses and pentoses(1-2). The organism has been grown on a synthetic medium containing glucose(3-5). However, when xylose replaced glucose in the synthetic medium, *L. pentosus* was reported not to grow(6). Growth was obtained when a supplement such as yeast extract was added and the existence of a "xylose factor" was postulated(6).

The present communication describes the study of an active principle in a liver extract supplement which promoted growth of *L. pentosus* on a synthetic medium containing either xylose or L-arabinose as a carbohydrate source.

Microbiological Methods. The organism used was *Lactobacillus pentosus,* Strain 124-2 (identical with *Lactobacillus plantarum* ATCC No. 8041). The synthetic medium employed was that described for the assay of nicotinic acid with *Lactobacillus arabinosus* (7) except that nicotinic acid was added and glucose was replaced by L-arabinose. Assays were carried out using either conventional microbiological technics in test tubes or by the pad plate technic(8) in which test solutions were added to filter paper discs and placed on agar plates seeded with *L. pentosus.* In the latter instance the diameter of the growth zone was used as a measure of the biological activity.

Results and Discussion. The data in Table I illustrate that *L. pentosus* failed to grow in a synthetic medium containing L-arabinose as a substrate unless a supplement such as liver extract or yeast was added. Small amounts of glucose duplicated the growth stimulatory effect of the liver extract. However, when the organism was cultured in the absence of L-arabinose, liver extract was inactive, and in experiment 1, for example, 1000 times as much glucose was now needed for growth.

Other sugars were tested for growth of *L. pentosus* on an L-arabinose medium. The data in Table II show that glucose and mannose were the most active while galactose and maltose had slight activity. Fructose and

TABLE I. Response of *Lactobacillus pentosus* 124-2 to Liver Extract, Yeast Extract, or Glucose in the Presence or Absence of L-arabinose.

Additions to 10 ml basal medium	Optical density after 18 hr incubation: 200 mg L-arabinose added per tube, Exp. 1	Exp. 2	L-arabinose omitted, Exp. 1	Exp. 2
0	.14	0	0	0
.01 ml liver extract	.66		0	
.03		.66		0
100 mg Difco yeast extract		.66		0
.001 mg D-glucose	.20	.06	0	0
.01	.41	.27	0	0
.10	.55	.40	0	0
1	.63	.49	.06	.06
10	.77	.65	.44	.46
100	.86	.78	1.05	1.10

TABLE II. Relative Activities of Various Carbohydrates in Promoting Growth of *Lactobacillus pentosus*, Strain 124-2, on an L-arabinose Medium, Using the Pad-Plate Method.

Substance	Relative activity (units/mg)
Water soluble liver fraction	1.0 ± 0
D-glucose	5.9 ± .4
mannose	5.2 ± .7
galactose	.3 ± .1
fructose	<.02
Maltose	.8 ± .2
Lactose	<.02
Sucrose	Inhibitory to glucose

One unit of activity is defined as the activity obtained from 165 μg of glucose under standard assay conditions.

lactose had little or no activity and sucrose was mildly inhibitory to glucose.

In order to determine whether the growth-stimulating activity of liver was due only to its sugar content or to yet another substance, the liver extract was fractionated, and the total "xylose factor" activity, solids and glucose content were measured. Glucose was determined by reduction of copper sulfate(9) and by periodate oxidation(10). The results of these experiments are summarized in Table III.

The "xylose factor" activity was not removed by cation or anion exchange resins nor was it adsorbed to any extent on charcoal. The ratio of the "xylose factor" activity to the glucose determined by both methods was relatively constant in the various fractions whereas the ratio of the activity to total solids varied.

The fractions having substantial amounts of "xylose factor" activity were treated with phenylhydrazine and in every case an osazone formed which had the characteristic crystal structure of glucosazone. The osazone from the charcoal effluent no. 2 was converted to the phenyltriazole. The melting point of the crystals was 193-194°, which is the melting point of phenyl-D-glucosotriazole(11).

Paper chromatograms were made with glucose and the liver extract using water-n-butanol-pyridine as a developer(12). The strips were cut lengthwise after development, and one half of each strip was treated with the sugar reagent 3-5-dinitrosalicylic acid(12), and the other half was bioautographed with *L. pentosus*. The single zone of microbiological activity coincided with the one area which gave the reaction for a sugar on each strip, so that the "xylose factor" activity in the liver extract was not separated from the sugar present.

In a personal communication, V. Shulimson and S. H. Hutner inform us that they have found that when *Streptococcus faecalis* is grown in media in which neutralized gluconolactone is substituted for glucose, a small amount of glucose or yeast autolysate becomes necessary. It remains to be determined whether the glucose content of this yeast preparation is responsible for the activity.

Summary. 1. *L. pentosus*, Strain 124-2, will grow on a synthetic medium containing L-arabinose as the carbohydrate source provided a small amount of glucose is added. 2. The supplement of glucose can be replaced by a liver extract. The active principle in the liver extract could not be separated by

TABLE III. Fractionation of the "Xylose Factor" Activity in Water-Soluble Liver Extract.

			Glucose (g)				
Fraction	Activity, units × 10^{-3}	Dry wt (g)	Copper sulfate reduction	Periodate oxidization	Units/mg dry wt	Units/mg glucose (reduction)	Units/mg glucose (oxidation)
70% alcohol filtrate of water soluble liver fraction	100	99	27	5.3	1.0	3.7	1.9
Cation exchanger effluent*	125	50	28	97	2.5	4.5	1.3
Anion exchanger effluent†	67	27	16	21	3.7	6.3	4.8
Charcoal column effluent							
No. 1	2.5	7.4	.8	3.4	.34	3.1	.74
2	15.4	5.8	4.7	4.2	2.7	3.3	3.7
3	13.2	4.1	3.6	3.8	3.2	3.7	3.5
4	10.0	1.9	2.2	2.3	5.3	4.5	4.4
5	4.5	.7	1.1	1.2	4.1	4.0	3.7
6	1.6	.5	.3	.6	2.7	5.3	2.7
7	1.2	.2	.2	.3	6.0	6.0	4.0
8	5.3	1.8	1.5	1.2	2.9	3.5	4.4
9	ca .7	.6	<.1	.9	1.2	—	—

* Amberlite IR-120(H).
† Amberlite IRA-400.

ion exchange on synthetic resins, charcoal adsorption or paper chromatography from the sugar present in the liver extract. The total activity in the liver extract could be accounted for as the sugar content. Glucosazone was isolated from an active fraction of liver extract.

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Poliomyelitis Viruses in Tissue Culture. IV. Protein-Free Nutrient Media in Stationary and Roller Tube Cultures.* (19823)

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The multiplication of poliomyelitis virus in roller tube cultures of human and monkey tissues is usually accompanied by degenerative changes in the developing outgrowth (cytopathogenic effect)(1-6). For such cultures, tissue fragments are imbedded in a plasma clot attached to the inner wall of a test tube and after incubation in the presence of a suitable medium in a rolled drum at 35°C for several days, cellular proliferation is usually quite marked. If poliomyelitis virus is added to the cultures at this time, subsequent cytopathic changes in the outgrowth serve as an *in vitro* indicator of virus proliferation.

The present report is concerned with the simplification of the medium in which cells grow out from the tissue fragments and in which virus may be grown in tube cultures. Also, in confirmation of Li and Schaeffer(7), and Scherer and Syverton(6), satisfactory results may be obtained with tubes which are not rolled. The nutrient media in current use for work with poliomyelitis virus tube cultures contain serum or embryonic extracts in addition to serum ultrafiltrate and isotonic salt solution(1-6). In the present study, protein-containing, cellular growth stimulants (serum or embryonic extract) have been successfully replaced by a protein hydrolysate (lactalbumin)[†] and the roller drums have been replaced by racks for holding the tubes on their sides with the fragments submerged in the nutrient medium.

Materials and Methods. The technic of preparing roller tube cultures of monkey testicular tissue as used in this laboratory has been described in detail, together with the behavior of selected strains of poliomyelitis virus in tissue culture(3,5). These conditions have been followed in the present experiments except that the acetone-extracted chick embryo (ACE) in the medium has been replaced by a lactalbumin enzymatic hydrolysate.[‡] The powdered hydrolysate (5 g) was mixed with Hank's salt solution without sodium bicarbonate (95 ml) and dissolved by autoclaving at 10 lb pressure for 10 minutes. The solution is stable when kept frozen, and presumably at 4° also. If a slight precipitation occurs upon thawing, it can be brought into solution by heating in lukewarm water. The pH of the solution is 6.8 and it is brought to 7.5 with sterile 0.1 N NaOH before using. The *complete medium* which is added to the imbedded tissue fragments during outgrowth of cells consists of Hanks-Simms solution (3 parts of Hanks' balanced salt solution plus 1 part of Simms' ultrafiltrate) 9 parts, and 5% lactalbumin hydrolysate, 1 part. After the addition of virus, a 1% lactalbumin hydrolysate solution is used, as this concentration is sufficient to maintain the cells during the subsequent observation period. In each of the experiments to be described half of the tubes were rolled in a drum(5) and half were held stationary on their side in a rack or in a cardboard carton which was slightly inclined, so that 1 ml of nutrient fluid would form a shallow layer and reach not over 2/3 of the distance from the bottom to the opening of the 6 x 150 mm test tube. The 6 to 10 tissue fragments were placed in a line on one side of the test tube as recommended by Youngner, Ward, and Salk(4). This was particularly useful in the present experiments to insure that all fragments were submerged in the fluid medium. All tubes were held at 35°. They were removed every few days for microscopic examination of the cultures and for changing nutrient fluids.

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[*] Aided by a grant from the National Foundation for Infantile Paralysis.

[†] Protein digests had already been used to stimulate cell proliferation in tissue culture in 1928(9).

[‡] The hydrolysate was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. A typical analysis, according to the producer, yielded total nitrogen 12%, amino nitrogen 7.3%, ash 3.6%, sodium chloride 0.9%, and moisture 6.8%.

TABLE I. Effect of Lactalbumin Hydrolysate on Growth of Fibroblasts and Their Destruction by Poliomyelitis Virus.

	Growth of fibroblasts		Extent of degeneration on 10th day*		
	4th day	6th day	10^{-2} virus	10^{-3} virus	10^{-4} virus
LACT.,† 0.5% final conc.	Luxuriant	Luxuriant	3+	1+	—
0.1%	Good	"	3+	1+	—
0.01%	Beginning	Good	2+	1+	—
Chick embryo extr.	"	"	2+	1+	—

* 10th day of exp., or 4 days after addition of virus. Extent of fibroblast degeneration is given in arbitrary units which have been defined(5). 10^{-2} virus indicates tissue culture Y-SK virus, diluted 1 to 100, added to the tubes on the 6th day; 10^{-3} virus, diluted to 1 to 1000; etc.

† LACT. = Lactalbumin hydrolysate.

Results. When lactalbumin hydrolysate is used in place of ACE, fibroblasts from monkey testicular tissue grow out more rapidly. By the 4th day after the fragments are implanted in the tubes, the outgrowth is sufficiently luxuriant so that the tubes may be inoculated with virus. As shown from Table I, this is about 2 to 3 days earlier than seen with ACE. Such tubes respond to tissue culture adapted virus in similar fashion to ACE-containing tubes, and titration results similar to those shown in Table I have been obtained with stationary as well as rolled tubes. Photomicrographs in Fig. 1 and 2 illustrate the clear nature of the normal fibroblasts and also the extensive degeneration produced by the action of the virus, in both rolled and stationary tubes. For purposes of comparison, tubes containing ACE were set up at the same time and typical fragments are also shown.

A number of such experiments have been carried out in which virus has been added 4 days after the tissue fragments have been implanted in the tube. With slower growing fragments (ACE medium), it has been customary to change fluid on the 3rd or 4th day after implantation and add virus on the 6th or 7th day. Because cells grow out faster with hydrolysate in the medium, virus may be added at the time of the first fluid change. This is particularly convenient in carrying out neutralization tests for poliomyelitis antibodies in tissue cultures, for the tubes need be handled only on the 4th day when fluid is changed and the virum-serum mixture is added, and on the 8th day when the tubes are read(5).

Virus growth was supported as well, and apparently for longer periods, in the hydrolysate tubes than in the ACE tubes. A typical experiment showing the titrations of fluids harvested at various intervals from ACE and lactalbumin hydrolysate cultures, both rolled and stationary, is shown in Table II. Although virus could still be detected in all tubes 30 days after their inoculation, virus production fell off markedly in the ACE after the 18th day and in the hydrolysate tubes after the 23rd day or perhaps even later. The degeneration observed in the hydrolysate tubes was specific, for not only was it prevented by homotypic antiserum, but also fluid from tubes showing degeneration produced a similar effect when titrated in ACE tubes. The degeneration produced in titrating the hydrolysate tubes was also proven to be specific. In tubes which yielded an endpoint at the 10^{-4} concentration, fluid was harvested from the last tubes in the series showing degeneration (10^{-4}) and from the tubes at the next dilution also (10^{-5}). These fluids were transferred to a new series of ACE roller tubes, half of which had each received 0.1 ml of 1:10 Brunhilde antiserum. The results, listed below, indicate that Brunhilde virus was present in the tubes at the titration endpoint (read by cytopathic changes), but not in the next tubes in the series which failed to show degenerative changes:

	Results of tests in ACE roller tubes, degeneration
Harvest from 10^{-4} tubes	4+
10^{-4} tubes + BA*	0
10^{-5} tubes	0
10^{-5} tubes + BA	0

* BA = Brunhilde antiserum.

After the 16th day or so, the fibroblasts in the hydrolysate control tubes tend to shrink or wander in toward the tissue fragment and

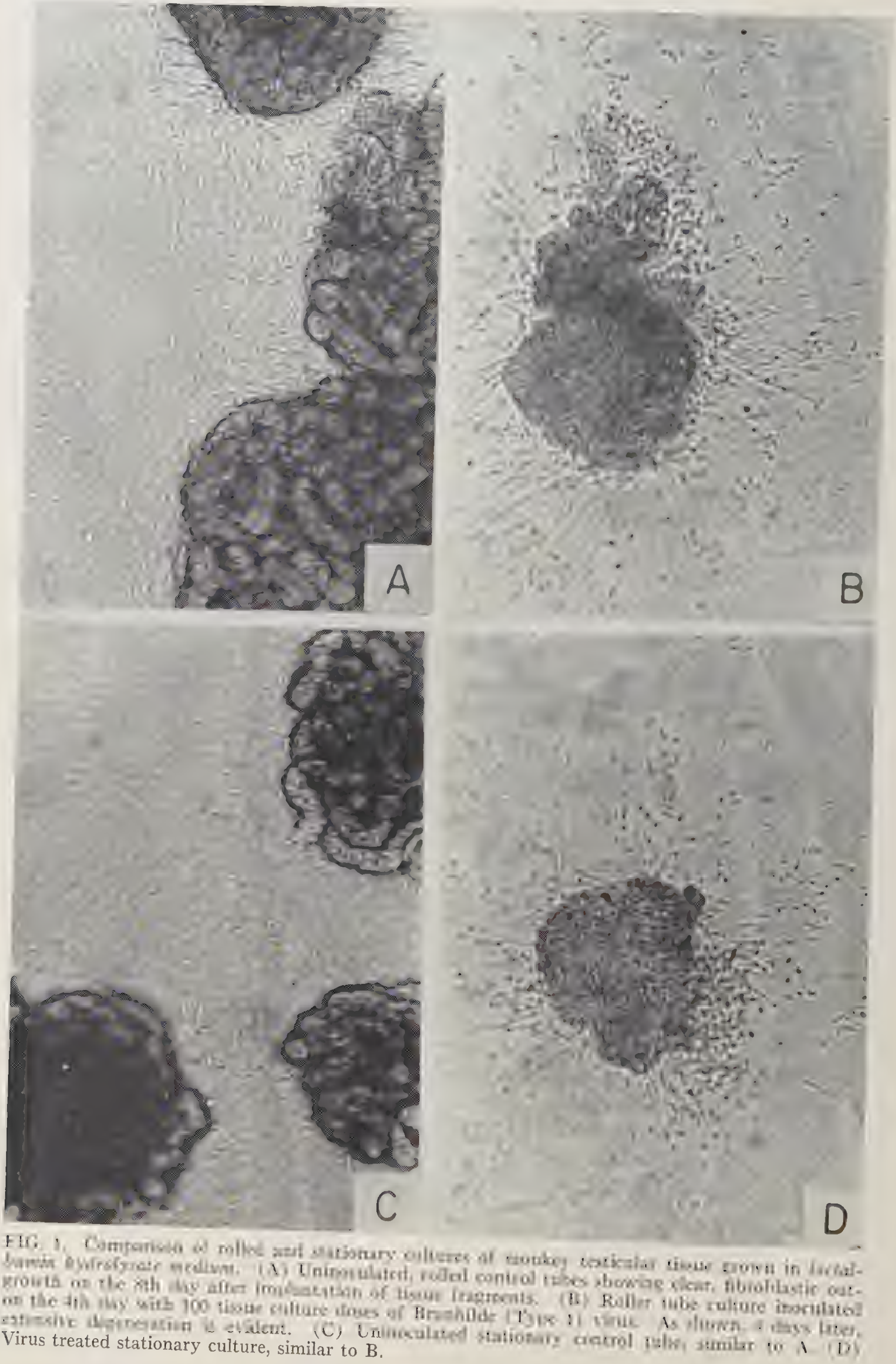

FIG. 1. Comparison of rolled and stationary cultures of monkey testicular tissue grown in *lactalbumin hydrolysate medium*. (A) Uninoculated, rolled control tubes showing clear, fibroblastic outgrowth on the 8th day after implantation of tissue fragments. (B) Roller tube culture inoculated on the 4th day with 100 tissue culture doses of Brunhilde (Type 1) virus. As shown, 4 days later, extensive degeneration is evident. (C) Uninoculated stationary control tube, similar to A. (D) Virus treated stationary culture, similar to B.

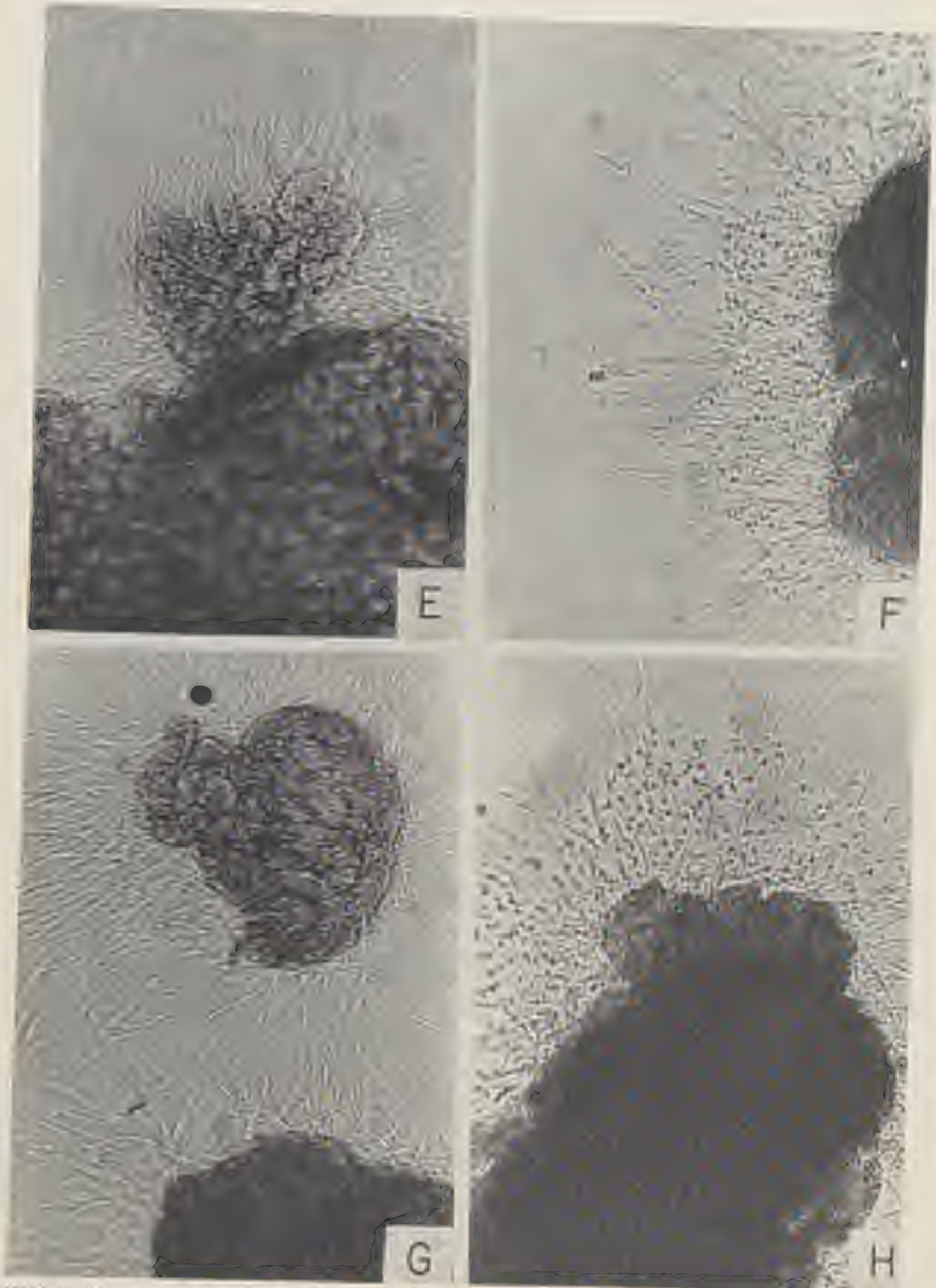

FIG. 2. Comparison of rolled and stationary cultures of monkey testicular tissue grown in *acetone-extracted chick embryo medium.* (E) Uninoculated, rolled control tubes showing clear, fibroblastic outgrowth on the 8th day after implantation of tissue fragments. (F) Roller tube culture inoculated on the 4th day with 100 tissue culture doses of Brunhilde (Type 1) virus. As shown, 4 days later, extensive degeneration is evident. (G) Uninoculated stationary control tube, similar to E. (H) Virus treated stationary culture, similar to F.

TABLE II. Titrations of Harvests from Stationary and Rolled Tubes Containing Acetone-Extracted Chick Embryo (ACE) or Lactalbumin Hydrolysate (LACT.)*

	Type tube culture in which virus was grown†			
Day of harvest	ACE rolled	ACE stationary	LACT. rolled	LACT. stationary
4	10^{-3}	10^{-3}	10^{-4}	10^{-3}
9	10^{-4}	10^{-3}	10^{-4}	10^{-3}
13	10^{-2}	10^{-1}	10^{-4}	
18	10^{-2}	10^{-2}	10^{-3}	10^{-2}
23	10^{-1}	10^{-1}	10^{-3}	10^{-3}
30	10^{0}	10^{0}	10^{-2}	10^{-1}

* All titrations were carried out in rolled tubes containing ACE, using 0.1 ml per tube as in inoculum, and serial 10-fold dilutions. Results are given as the least concentration of fluid producing a cytopathogenic effect in at least one of two tubes during an observation period of 8 days. For further details see Ledinko, Riordan, and Melnick(5).

† Three tubes of each type indicated were inoculated with 100 tissue culture doses of Brunhilde (Type 1) virus, 8th tissue culture passage. Fluids from each set of three tubes were pooled when harvested.

parallel the periphery of the fragment. Such fibroblasts are not suitable for microscopic examination, which must be discontinued. However, the control tissue continues to metabolize well for over 30 days, at this time still bringing the pH of the medium from 7.5 to 7.0 in a few days. As shown in Table II, virus continues to multiply for sometime after the fibroblasts have degenerated. This is true for stationary as well as rolled tubes.

Several thousand tubes containing lactalbumin hydrolysate in the medium have been used in this laboratory, in which fibroblastic outgrowth was obtained in stationary tubes.

Discussion. This study indicates that the presence of protein in the *fluid* phase of the nutrient medium is not essential for the growth of fibroblasts or for the multiplication of poliomyelitis viruses in tube cultures. Thicke *et al.*(8) have recently found that synthetic medium "199"(10) will support the propagation of poliomyelitis virus in flask cultures, and Salk *et al.*(11) have also indicated that it is highly satisfactory in roller tube cultures. Future work will undoubtedly simplify the medium so that it will contain only those compounds essential for outgrowth of cells and for virus multiplication. The use of commercially available and inexpensive lactalbumin hydrolysate offers certain advantages in this direction, particularly as a substitute for the amino acid block used in synthetic media(10).

The benefits of protein-free media are great in subsequent work with tissue culture grown virus. To mention only a few: (i) foreign proteins can be eliminated from vaccines prepared from such fluids (except for the small amounts coming from the plasma clot and the tissue fragments), and (ii) complement-fixing antigens and purified virus preparations should be more easily obtained from starting material in which extraneous protein is absent or present in insignificant amounts. In their work on the preparation of complement-fixing antigens, Svedmyr, Enders, and Holloway(12) have recommended the replacement of the protein-containing medium by a dialyzable medium, but only after cellular outgrowth had occurred and after inoculation of the cultures with virus.

Stationary tubes are particularly useful in allowing poliomyelitis virus isolations, typing, and neutralization tests to be carried out in laboratories in which roller drums are not available. If tubes with tissue fragments imbedded in a plasma clot become available from a central supply agency, then any good hospital laboratory equipped with an incubator should be able to carry out diagnostic tests for poliomyelitis.

Summary. Poliomyelitis virus may be propagated in test tube, plasma-clot cultures of monkey testicular tissue in the absence of protein in the fluid phase of the nutrient medium. The tubes may be held stationary with the fragments submerged under a thin layer of medium, or they may be turned in a roller drum. The medium used contained lactalbumin enzymatic hydrolysate, serum ultrafiltrate, and balanced salt solution. With such a medium, fibroblasts grow out within 4 days after the cultures are made, and are then suitable for use in virus titrations and neutralization tests in which the presence of virus is indicated by its cytopathogenic effect on the fibroblasts. Also such cultures continue to produce virus for a period of about four weeks, long after the degeneration of the fibroblasts.

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Ineffectiveness of Aureomycin and Terramycin Against Rabies Street Virus in Mice.[*] (19824)

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There is no therapeutic material that is efficacious against infection with rabies virus. Therefore it was of interest to try terramycin for two reasons: a) concentration of ingested terramycin in the brain is reported to be relatively high(1), and b) conflicting evidence has been presented on the efficacy of terramycin against rabies virus. Quilligan *et al.*(2) showed no effect of terramycin on the titration of rabies virus in mice, while Azevedo and de Macedo(3) claimed 98% survival rates in rats and rabbits. This work was undertaken to secure more evidence on the action of terramycin on the rabies virus, and to compare it with aureomycin.

Method and materials. The virus material was rabies street virus in a 20% suspension of dog salivary gland.[‡] The LD_{50} of this virus by intracerebral inoculation in 3-week-old white mice was $10^{-5.8}$ as calculated by the Reed-Muench method(4). The terramycin and aureomycin were incorporated into finely ground fox-chow at the rate of 100 mg of antibiotic per 100 g of feed. The mice were starved for a day prior to the experiment so that they would accept the ground mixture. In order to get some idea of how much drug the mice were ingesting, a weighed amount of feed and material was placed in a Petri dish and the material was weighed each day. The difference was then taken to be the amount consumed by the mice in the cage. The amount of aureomycin or of terramycin actually ingested by mice under this regimen is adequate to ensure a definite therapeutic response with murine and scrub typhus infections(5).

Procedure. Twenty mice divided into 2 cages with 10 mice in a cage were placed on the aureomycin feed mixture. Twenty mice divided into 2 cages with 10 mice in a cage were placed on the terramycin feed mixture. Twenty mice divided into 2 cages with 10

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[†] Scientist, U. S. Public Health Service.

[‡] This material was furnished by Dr. Koprowski and Dr. Cox of Lederle Laboratories, American Cyanamid Co.

TABLE I.

		Avg day of death	Stand. dev.
Terramycin	Cage I	12.3	1.9
	" II	12	1.8
Aureomycin	" I	12.1	2.2
	" II	11.9	1.5
Controls	" I	12.1	1.7
	" II	11.8	2.4

mice in a cage were used as controls. All 60 mice were inoculated in each masseter muscle with 0.05 ml of a 10^{-3} dog salivary gland suspension whose LD_{50} was measured by intracerebral titration was $10^{-5.8}$. Therefore each mouse received 0.1 ml of the salivary gland suspension or about 1200 intracerebral LD_{50} doses. The salivary gland was diluted with 10% inactivated rabbit serum in buffered saline solution (rabbit serum heated at 50°C for 15 minutes; the saline was buffered at pH 7.4). A second experiment was set up to determine the effect of contact of terramycin on rabies virus. The salivary gland suspension was diluted with sterile distilled water suspension of terramycin so that each cubic centimeter of final salivary gland suspension had 300 gamma of terramycin per ml. The final pH of this solution was 3.8. Control salivary gland suspensions without terramycin were made up to pH 3.8. Both the terramycin-salivary gland suspension and the control suspensions were incubated at 37°C for 2 hours. The final salivary gland concentration in both suspensions was 10^{-3}. Twenty mice were inoculated with the terramycin-salivary gland suspension at the rate of 0.05 ml into each masseter muscle. The 20 mice were divided into 2 cages of 10 each. The salivary gland suspension at pH 3.8 without terramycin was given to 20 other mice and these two were divided into two cages of 10 each. The observation period was 30 days. Each mouse that died was autopsied and the brain examined by means of impressions stained with Seller's stain and searched for Negri bodies.

Results. All Table I mice in the therapeutic trials died. Table shows average day of death in each category, 10 mice in each experiment.

All mice when autopsied showed Negri bodies when impression smears of the brain were stained with Seller's stain and examined microscopically.

The quantity of antibiotics ingested was roughly in the order of 200 γ per mouse per day. On the average the mice weighed approximately 15 g each and were predominantly males.

In the experiment where the terramycin was mixed with the virus suspension 4 mice died within the observation period of 30 days. Two of the controls died. Impression smears made of the brains of these animals showed Negri bodies when stained with Seller's stain. The survivors which were sacrificed at 31 days showed no Negri bodies.

Discussion and summary. 1. Neither aureomycin nor terramycin administered as described had an effect on the course of rabies in white mice when street virus was inoculated into the masseter muscle. 2. The pH of the terramycin preparation tried was sufficiently low to exert a lethal action on the street virus. A control suspension buffered at the same pH as the terramycin solution exerted the same lethal effect on the street virus.

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In vitro Action of Aureomycin on Oxidative Phosphorylation in Animal Tissues. (19825)

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In a preliminary report(1) from this laboratory, 0.0001 M aureomycin was shown to inhibit strongly the respiration of rat liver homogenates. Loomis(2), using a washed rabbit kidney residue preparation, found that only phosphorylation, but not oxidation, was inhibited by aureomycin at a similar concentration. We have found that the respiration of washed rat liver mitochondria is also inhibited under certain conditions. Using the suspending medium of Kielley and Kielley(3), we also have found that aureomycin had only the effect reported by Loomis. This report is mainly concerned with the conditions and factors which are associated with the inhibition of oxidation of rat liver mitochondria by aureomycin.

Experimental. Normal rat liver mitochondria were prepared according to the method of Schneider(4) using 0.25 M sucrose. The washed sedimented mitochondria were homogenized in 0.154 M KCl to give a suspension containing the equivalent of 1 g of liver per milliliter. All operations were carried out in a refrigerated centrifuge at 0° or in an ice bath. The washed liver mitochondria (500 mg liver equiv. except where noted) were added to Warburg flasks containing the suspending medium as noted in the respective tables and the oxygen consumption was determined. The center well contained 0.2 ml of 2 N NaOH and a 2 sq cm filter paper wick. When inorganic phosphorus was to be determined, the respiration flasks were quickly removed, placed in an ice bath, and the contents transferred to cold centrifuge tubes containing 0.3 ml of 30% trichloroacetic acid. The flasks were rinsed with 1 ml of water and the washings were added to the incubated mitochondrial suspension. The inorganic phosphorus was estimated in the TCA filtrate by the Fiske-SubbaRow method(5). The inorganic phosphorus uptake was determined as the change in inorganic P from the end of the thermal equilibration period (zero time) to the end of the incubation.

Results. The influence of phosphate concentration, ageing of mitochondria, and mag-

TABLE I. Influence of Phosphate Concentration on Oxidative Phosphorylation by Washed Rat Liver Mitochondria.

	10 μm phosphate/flask			30 μm phosphate/flask		
Addition	Uptake of oxygen in μm	Changes in μm phosphate	Phosphorus-oxygen ratio	Uptake of oxygen in μm	Changes in μm phosphate	Phosphorus-oxygen ratio
Control	3	+ .8		2.5	+ 1.6	
24 μm AMP	3.2	—5.5	1.7	3.8	—10.6	2.8
6 " ATP	5.3	+ .4		4.6	+ 1.4	
24 " AMP + 6 " ATP	3.4	—7.1	2.1	6.3	—10.5	1.7
.4 " A*	3.2	+1.1		1.3	+ 1.4	
24 " AMP + .4 " A	6.5	+1.2		7	— .6	
6 " ATP + .4 " A	1.5	+9.6		2.6	+ 9.3	
24 " AMP + 6 " ATP + .4 " A	5.2	+5.1		4.5	+ 5.3	

Flask contents: 15 μm $MgCl_2$, 120 μm KCl, 0.04 μm Cytochrome C, 30 μm α-ketoglutarate, 300 mg equiv. liver mitochondria, 0.2 ml of 2N NaOH in center well, H_2O to make 3.0 ml. Incubation tem.: 36.6°C. Incubation time: 20 min. after thermal equilibration. All values are means of 4 or more determinations. * A = Aureomycin.

TABLE II. Effect of Aging on the Aureomycin Inhibition of Oxidative Phosphorylation by Rat Liver Mitochondria in the Absence of Added Magnesium.

Aureomycin per flask in μm	Before aging: Uptake of oxygen in μm	Before aging: Changes in μm phosphate	Before aging: Phosphorus-oxygen ratio	After 2 hr aging at 0°C: Uptake of oxygen in μm	After 2 hr aging at 0°C: Changes in μm phosphate	After 2 hr aging at 0°C: Phosphorus-oxygen ratio
.0	6.5	12.8	1.8	6.2	6.4	1
.1	6.1	8.8	1.4	4.5	4.1	.9
.2	4.6	4.5	1	3.4	5	1.5
.4	3.6	3.9	1.1	3.2	3.5	1.1
.8	1.2	2.3	1.9	2.4	3.2	1.3

Flask contents: 125 μm KCl, 30 μm phosphate pH 7.4, 0.04 μm Cytochrome C, 12 μm adenylic acid, 30 μm α-ketoglutarate, 500 mg equiv. liver mitochondria, 0.2 ml 2 N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time: 30 min. after thermal equilibration.

nesium ion, on the aureomycin inhibition of oxidative phosphorylation are shown in the accompanying tables.

The data in Table I indicate that the rat liver mitochondrial system is capable of considerable phosphorus uptake as well as oxidation when adenylic acid is present. In all cases where aureomycin is present there is but little or no uptake of phosphorus. However, aureomycin inhibits oxygen uptake only when ATP is substituted for AMP. This inhibition of respiration appears to be greater at the lower concentration of inorganic phosphate.

In another experiment the magnesium chloride was omitted from the suspending medium (Table II) and the effect of increasing levels of aureomycin and ageing the mitochondria at 0°C is shown. Here the inhibition of respiration increases with the aureomycin concentration. It should also be noted that the phosphorylating ability of the mitochondria is also reduced by ageing at 0°C, but that the oxidative capacity is little altered. This loss in phosphorylating activity with ageing is similar to that found by Kielley and Kielley (3) in mouse liver mitochondria, aged at 28°C.

When magnesium is added to aged mitochondria the inhibitory effect of aureomycin on respiration is reversed as shown in Table III.

The reversing effect of magnesium on the aureomycin inhibition of respiration is again shown in Table IV. Ageing appears to have little influence on the magnesium reversal of the aureomycin inhibition. In this table it is noted that loss of respiratory activity accompanies ageing. The magnitude of effect of ageing on different preparations varies considerably.

TABLE III. Effect of Magnesium and Aureomycin on Oxidation of α-ketoglutarate by Rat Liver Mitochondria Aged 2½ Hr.

Aureomycin per flask in μm	Oxygen uptake in microatoms, Magnesium per flask in μm: 0	.4	4
.0	6.4	5.1	5.5
.2	4.9	5.5	5.6
.4	3.2	2.2	5.2

Flask contents: 0.04 μm cytochrome C, 12 μm adenylic acid, 125 μm KCl, 30 μm phosphate pH 7.4, 30 μm α-ketoglutaric acid, 500 mg equiv. liver mitochondria aged 2½ hr at 0°C, 0.2 ml 2N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time, 30 min. after thermal equilibration.

TABLE IV. Influence of Aging on Aureomycin Inhibition of Respiration of Rat Liver Mitochondria in the Presence and Absence of Magnesium.

Additions per flask	Oxygen uptake in microatoms, Aging time at 0°C: 0	1 hr	2 hr
Control	9.2	11.0	4.7
15 μm $MgCl_2$	5.5	7.5	2
.4 " A*	7.7	7.6	0
15 " $MgCl_2$ + .4 μm A	11.9	12.9	3.5

Flask contents: 12 μm adenylic acid, 125 μm KCl, 0.04 μm cytochrome C, 30 μm phosphate pH 7.4, 30 μm α-ketoglutarate, 500 mg equiv. liver mitochondria, 0.2 ml 2N NaOH in center well, H_2O to make 3 ml. Incubation temp., 19.9°C. Incubation time, 30 min. after thermal equilibration.

* A = Aureomycin.

Discussion. It is well established that aureomycin inhibits phosphorylation associated with oxidation of citric acid cycle substrates under certain conditions. Previous reports(2,6) of the *in vitro* action of aureomycin have been based upon systems which were heavily fortified with magnesium, phosphate and fluoride. It has been shown in the present work that the inhibitory effect of aureomycin on oxidation does not appear when high concentrations of magnesium and phosphate are present. In unpublished experiments of this laboratory it has been found that fluoride is also antagonistic to the aureomycin effect on oxidation. It is conjectured that the effect of ageing of mitochondria is to release endogenous magnesium from the centers of enzyme activity into the suspending medium. This would account for the fact that aureomycin is frequently ineffective in reducing the oxygen uptake when fresh mitochondria are used. Aureomycin has been found to form complex metal salts very readily and among them is a very insoluble magnesium salt. Thus if the magnesium concentration of the active centers is low, the addition of aureomycin could be expected to reduce the oxygen uptake by holding the remaining magnesium in an inactive complex. The addition of exogenous magnesium would restore the oxygen uptake. Our studies would seem to support this hypothesis.

In a recent paper(7) on the regulation of the rate of oxidation in rat liver mitochondria Potter and Recknagel examined the relationship of 2, 4-dinitrophenol (DNP) on oxidation and apparent ATP-ase. Their data using DNP, strikingly resembles our results with aureomycin when sufficient magnesium is present in the medium. Aureomycin in a similar system will accelerate the breakdown of ATP, but instead of increasing the oxygen uptake, decreases it or is without effect depending upon the activity of the individual preparation. However, if AMP is substituted for ATP the identical effect found by Potter with DNP and ATP is shown by aureomycin. One would expect that the aureomycin effect on oxidation would be the same using either AMP or ATP, if the only effect of aureomycin were to accelerate the breakdown of ATP. It is believed that this point would bear further investigation. Lardy in a recent review(8) on the influence of inorganic ions on phosphorylating reactions points out that, "although detailed studies have been made of the ion requirements of various oxidizing systems, the requirement for magnesium has been so unquestionably accepted that no studies have been reported on the mode of action." It is evident from the data we have presented that the role of magnesium in oxidative phosphorylation deserves more investigative attention.

It should be emphasized that the inhibitory effects of aureomycin on oxidation and phosphorylation were noted at a lower limit of about 10^{-4} molar. This concentration is quite high when compared to 10^{-8} molar required for inhibition of growth of susceptible microorganisms. Thus, one might argue that these inhibitory *in vitro* effects of aureomycin ae not necessarily related to its antibiotic activity.

Summary. 1. The inhibition of oxidative phosphorylation in animal tissue by aureomycin is confirmed. 2. In the presence of magnesium, low phosphate, and ATP, aureomycin inhibits oxidation by washed rat liver mitochondria. If AMP is substituted for ATP, no inhibition is found. 3. In the absence of magnesium, the inhibitory effect of aureomycin on respiration is proportional to its concentration. Ageing the mitochondria at 0°C appears to accentuate this inhibition. 4. Using aged mitochondria and AMP, the addition of magnesium reverses the aureomycin inhibition of respiration.

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Particle Size of the MEF_1 Lansing Strain of Poliomyelitis Virus. (19826)

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There is no uniformity of opinion about the morphology of the viruses of the poliomyelitis group. Particle sizes have been determined frequently, but the results reported are widely divergent. Thus using ultrafiltration technics Theiler and Bauer(1) found the size of poliomyelitis virus to be 10-15 mμ. This diameter approximates that found by Elford, Galloway and Perdrau(2) of 8-12 mμ. Theiler and Gard(3) established a diameter of 9-13 mμ for Theiler's virus, and Sanders and Jungeblut (4) found Columbia SK virus to be similar in size. Using other physico-chemical methods Gard and Pedersen(5) estimated the size of Theiler's virus to be 640 x 14 mμ. Tiselius and Gard(6) and Gard(7) found filamentous particles with a diameter of 15-20 mμ on examining purified Theiler's virus in the electron-microscope. Similar structures were found by Jungeblut and Bourdillon(8) in fluid from tissue cultures in which sk virus was propagated, and in purified preparations from mouse brain virus these authors found spherical particles with diameters ranging from 25-30 mμ. Loring, Schwerdt and Marton(9) reported a particle size of 15-20 mμ for purified MV strain of poliomyelitis virus. Particles of a similar magnitude were observed by Loring(10) in preparations of Lansing and Y-SK strains of poliomyelitis virus. Recently Leyon, Gard and Eklund(11) reported measurements made on electron photomicrographs of purified Theiler's virus. The most reliable measurements indicate that the elementary particles of Theiler's virus are approximately spherical and have a diameter of 28 mμ. Reagan, Shenk and Bruecker(12) published electron photomicrographs of virus particles extracted from monkey cords infected with the Leon strain of poliomyelitis. From their photographs the elementary particle appears to be a short rod. The width is 12-15 mμ and length 30-35 mμ. In view of the controversial results obtained by previous workers it was decided to reinvestigate the particle size of poliomyelitis virus with the ultracentrifugation technic of Polson and Linder(13).

Material and methods. The MEF_1 Lansing strain of poliomyelitis adapted to suckling mice by repeated intracerebral passage was used (Casals, Olitsky and Anslow(14), and Selzer, Sacks, and van den Ende(15)). At the time the virus was used in these experiments it had undergone 34 serial transfers in suckling mice. Its identity was confiramed by complement fixation and neutralization tests with a Lansing immune serum supplied by Dr. Bodian, Johns Hopkins Hospital. For centrifugation a 10% emulsion of brains from virus infected suckling mice was used. The suspensions were made in phosphate buffer at pH 8.2 and clarified by centrifugation for 1 hour at 2800 r.p.m. in a refrigerated horizontal centrifuge. The slightly opalescent supernatant fluid was placed in a graduated spinco angle centrifuge tube and the height of the meniscus noted. It was then spun in a rotor which had previously been brought to equilibrium temperature for the required speed. The total time of centrifugation included the period of acceleration and deceleration. The average speed of centrifugation is obtained by dividing the total number of revolutions by the total time. Immediately after the rotor stopped the temperature of water in the tube used for balancing the virus suspension was determined and the tube containing the virus then transferred to a metal stand at the bottom of a large container of water at the same temperature as the centrifuge rotor, care being taken that the fluid water in the container was at a level above that of the meniscus in the centrifuge tube. With a special sampling device the fluid in the tube was removed in successive layers of 1 cm. Thus the contents of the tube was usually divided into 6 samples. Each sample was titrated by intracerebral inoculation of 10-fold dilutions into 3 to 4 weeks old mice. Fifty percent end-points were determined by the method of Reed and

TABLE I. Ulracentrifugation of Poliomyelitis Virus, MEF_1 in 1% *C. sincta* Haemocyanin.

r.p.m.	t_2–t_1 in min.	T°C	η/η_{20}	LD$_{50}$ of samples at— 1 cm	2 cm	3 cm	4 cm	5 cm	Bottom	LD$_{50}$ of original material
30000	105	12	1.24	0	T*	0	T*	0	—	4
22840	100	9.5	1.32	2.1	1.6	2	2.5	2.5	4.5	4.6
20000	100	9	1.345	1.6	.8	1.7	1.6	3	3.7	4

* T = Trace.

$\alpha = 26°$; $x_1 = 5.31$ cm

Muench(16). The titres of the different samples were plotted against the relative positions in the centrifuge tube. The position of the sedimenting virus boundary in the centrifuge tube, which corresponds to the 50% concentration point in the Svedberg light absorption technic is determined from the position of a line drawn through a point representing a titre T, where

$$T = \log\left(\frac{\text{antilog } T_2 - \text{antilog } T_1}{2}\right) \qquad (1)$$

and T_2 is the virus titre below, and T_1 that of the virus above the boundary zone respectively. From the position of the sedimenting boundary thus determined the sedimentation constant is calculated using the equation of Svedberg and Pedersen:

$$S = \frac{2\text{ H Sin }\alpha}{(2\text{ X}_1 + \text{H Sin }\alpha)\ \omega^2\ (t_2 - t_1)}\ \eta/\eta_{20} \qquad (2)$$

where X_1 = distance of initial boundary from the axis of rotation, α the angle of inclination of the tube with the axis of rotation, ω the angular velocity of the centrifuge rotor and $(t_2 - t_1)$ the time of centrifugation. X_1 the distance of the meniscus from the centre of rotation must be corrected for errors due to distortion which the tube undergoes during centrifugation. η/η_{20} is the ratio of viscosities of the solution at the temperature of centrifugation to that of water at 20°C.

To minimize heat convection currents during and after ultracentrifugation the virus was centrifuged in the presence of approximately 1% of whelk (*Caminella sincta*) haemocyanin. On centrifuging haemocyanin it was noticed that below the sedimenting boundary a protein gradient formed which had a function of overcoming heat convection disturbances similar to that of the sugar gradient used in the centrifugation experiments of Pickles(17).

Results. The results of experiments conducted at different rotor velocities are recorded in Table I.

The haemocyanin used in this experiment had a sedimentation constant of 100 Svedberg units and at 20000 r.p.m. for 100 min., its boundary moved 2.7 cm down the tube. From the above table it is evident that the virus boundary had reached the bottom of the tube at rotor velocities greater than 22840 r.p.m. At 20000 r.p.m. the boundary did not extend down the full length of the centrifuge tube but reached a level between 3.5 and 5.5 cm below the initial meniscus. The existence of this boundary is indicated by the sudden increase in the titres of the samples taken below the 3.5 cm level. From equation (2) it can be shown that the sedimentation constant of the virus lies between 129 and 189 Svedberg units. The most likely position which the boundary will occupy in the tube is that which can be calculated from equation (1).

This boundary is indicated by the vertical interrupted line in Fig. 1. This line cuts the

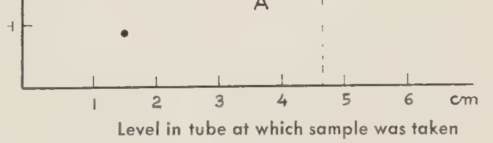

FIG. 1. Titres expressed in neg log LD$_{50}$ of samples taken at different levels in centrifuge tube of virus spun at 20000 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD$_{50}$.

TABLE II.
Ultracentrifugation Experiments of Virus Suspension Clarified by Different Methods.

Suspension	LD$_{50}$ of samples taken at the following levels 1 cm	2 cm	3 cm	4 cm	5 cm	6 cm	LD$_{50}$ of material used at the start	S$_{20}$
After centrifugation only	1.2	1.3	2.1	3	3.3	4	4.3	183
After centrifugation and ether extraction	T*	T*	T*	2.8	2.9	4.2	3.7	170
After centrifugation, ether extraction and tryptic digestion	0	0	1.2	1.2	<2	4	3.4	>208

* T = Trace.

$x_1 = 5.31$ $a = 26°$ r.p.m. 19740 time = 100 min. T°C = 9 $\eta/\eta_{20} = 1.345$

abscissa at 4.65 cm from the initial meniscus. The sedimentation constant calculated from this value is 163.1 Svedberg units which is in agreement with the value of 160-170 S reported by Gard and Pedersen(5) for Theiler's virus. From this value of the sedimentation constant the particle size was calculated using the modified Stokes equation

$$r^2 = \frac{9}{2} \frac{S\eta}{(d-\rho)}$$

where r is the radius of the particle, S the sedimentation constant, d the density of the particle assumed to be 1.33 g/cc η the viscosity and ρ the density of the dispersion median. The calculated size of 29.8 mμ or approximately 30 mμ is in agreement with that found on electron micrographs of SK virus, Jungeblut and Bourdillon(8) and Theiler's virus Leyon, Gard and Eklund(11). Owing to the dependency of the above sedimentation constant on the virus titrations in mice, no great precision is claimed for this value of the particle size of the MEF$_1$ strain of poliomyelitis. However, from the result of experiments, such as those marked in Fig. 1 it can be concluded with confidence that the sedimenting boundary must lie within the range of changing virus concentration. (Portion A B of graph) From the position of A and B it can be calculated that the size of the virus particle is within the range of 26 to 32 mμ.

To establish that the poliomyelitis virus does not exist as a smaller particle attached to lipoid or other brain components, experiments were performed on infected mouse brain extracts previously clarified by ether extraction or by ether extraction and tryptic digestion.

MEF$_1$ infected mouse brains were emulsified in 1/15 M phosphate buffer at pH 8.1 and clarified by centrifugation at 2800 r.p.m. for 1 hour in a refrigerated centrifuge. The supernatant was then shaken up in an equal amount of anaesthetic ether in the cold and again centrifuged at 2800 r.p.m. for an hour in the refrigerated centrifuge. The aqueous layer was removed, placed in a vacuum and the major portion of the dissolved ether thereby removed. The residual ether was removed by dialysis overnight against a large volume of buffer at pH 8.1. The solution was divided into two equal volumes. To one portion crystalline trypsin, 0.01 g per 20 ml brain extract was added and allowed to digest at 37°C for 30 min., after which 4 ml normal rabbit serum was added immediately to inhibit further tryptic action. The solution was slightly opalescent after ether extraction but cleared completely after 15 minutes digestion with trypsin.

Separate experiments were carried out to determine the proteolytic activity of the trypsin used. After ether treatment, but before tryptic digestion, the protein content of the virus solution was 0.296 g/100 ml. After 15 and 30 minutes tryptic digestion it was 0.205 g and 0.195 g/100 ml respectively.

The following samples were then centrifuged simultaneously in the Spinco angle centrifuge, each in the presence of 1% whelk haemocyanin. 1) Brain emulsion clarified by centrifugation at 2800 r.p.m. for 1 hour. 2) Brain emulsion clarified by centrifugation and

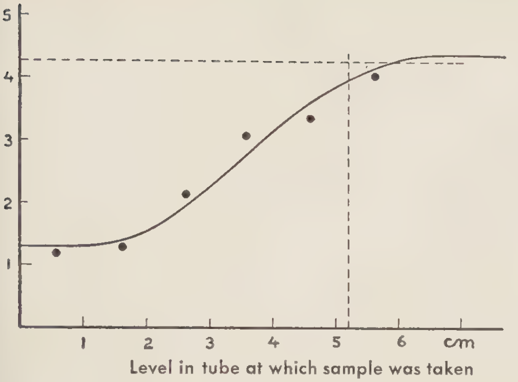

FIG. 2. Titres expressed in neg log LD_{50} of samples taken at different levels in centrifuge tube of virus spun at 19740 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD_{50}.

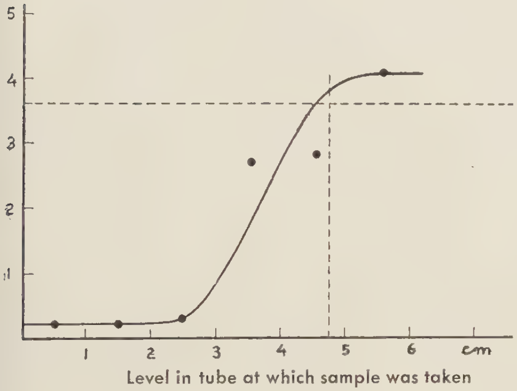

FIG. 3. Titres expressed in neg log LD_{50} of samples taken at different levels in centrifuge tube of virus after ether extraction and spinning at 19740 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and the vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD_{50}.

ether extraction. 3) Brain emulsion clarified by centrifugation, ether extraction and tryptic digestion. Successive layers were then removed from each of the tubes, and separately titrated for virus in order to determine the level of the sedimenting virus boundary. The results are recorded in Table II.

In all cases the haemocyanin boundary had moved down 2.7 cm. In Fig. 2 and 3 are recorded the virus titres (LD_{50}) of the different samples plotted against the levels in the centrifuge tubes from which the samples were taken. The vertical interrupted lines indicate the calculated position of the sedimentation boundaries.

In the case of ether treated suspensions less residual virus remains in the samples taken above the sedimenting boundary Fig. 3 than in the case of untreated suspensions. This observation had been confirmed in subsequent experiments. This may indicate that lipoids in untreated suspensions by adhering to the wall of the centrifuge tubes impedes the downward movement of the virus along the sides of the tubes during centrifugation.

It appears that the virus treated with ether and trypsin has been almost completely sedimented whereas in the other samples the virus has not reached the bottom of the tubes. There is certainly no evidence that ether treatment and tryptic digestion reduces the size of the infective virus particle as the sedimentation constant and consequently the particle size is again of the same order of magnitude as found in the previous experiment.

Summary and conclusions. 1. Using a Spinco preparative ultracentrifuge (Model L) a sedimentation constant of 163 S has been determined for the MEF$_1$ strain of poliomyelitis virus adapted to suckling mice. This sedimentation constant corresponds to a particle size of approximately 30 mμ when a particle density of 1.33 g/cc is assumed. This size is larger than that previously reported especially by investigators using ultrafiltration technics. 2. The high sedimentation constant is apparently not due to adsorption of the virus to tissue fragments. Thus preliminary clarification by ether and tryptic digestion did not reduce the speed of sedimentation of the virus during centrifugation. On the contrary such preliminary clarification, if anything, appeared to have increased the sedimentation constant of the virus. 3. The size calculated from results of the centrifugation experiments are in agreement with those obtained from electron photomicrographs and are probably closer to the correct value than those obtained by ultrafiltration.

We would like to thank Prof. M. van den Ende for his constant interest and helpful criticism during the course of our work.

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Bioautographic Analysis of Blood for Growth Factors* Active for *L. leichmannii* 4797. (19827)

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Previous observations by Couch *et al.*(1) on vit. B_{12} content of blood from various species have shown that the vit. B_{12} activity of turtle, chicken and turkey blood is higher than that of other species with the exception of the rabbit. These workers reported further that autoclaving blood samples from the chick, turkey and turtle at pH 12 destroyed only a part of the *L. leichmannii* activity. Such activity was explained on the basis of the fact that the latter species have nucleated erythrocytes and that the *L. leichmannii* response of the alkali treated and autoclaved blood samples was due to thymidine. A later study (2) reported values on vit. B_{12} content of blood from various species which were within the range of those reported by Couch *et al.*(1). The B_{12} content of blood from different breeds of cows has been determined in this laboratory(3). Blood samples from the Jersey breed contained the least B_{12} while those from Guernsey, Holstein and Brahman breeds were significantly higher.

The present study was designed to determine whether blood from species with nucleated erythrocytes contained growth factors for *L. leichmannii* 4797 other than vit. B_{12}.

Experimental. Five hundred ml of whole blood was collected from the cow, sheep and pig in oxalated containers. Blood samples from the chick, turkey and turtle were each pooled in order to obtain 500 ml of whole blood. In all cases blood samples were diluted with an equal volume of water, layered with toluene and incubated at 37°C for 24 hours. The autolysates were adjusted to pH 7 and autoclaved for 5 minutes at 120°C. After cooling, the samples were homogenized, centrifuged and the centrifugates were concentrated *in vacuo* to 20 ml. The vit. B_{12} content of each autolyzed blood sample (prior to concentration *in vacuo*) was determined according to the titrimetric method of Skeggs *et al.*(4) with *Lactobacillus leichmannii* 4797 as the test organism and crystalline vit. B_{12} as the standard. A 10 ml portion of each of the

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autolyzed blood concentrates (prepared as described above) from the chick, turkey and turtle blood were adjusted to pH 12 and autoclaved for 1 hour at 120°C. After cooling the samples were readjusted to pH 7 and concentrated *in vacuo* to a volume of 10 ml. Other blood samples were obtained by pooling 500 ml samples of fresh whole blood from the chick, turkey and turtle separately, mixing with an equal volume of water and autoclaving immediately for 15 minutes at 120°C. After cooling, the samples were layered with toluene, incubated at 37°C for 24 hours, reautoclaved at 120°C for 5 minutes. After cooling the samples were homogenized, centrifuged and the centrifugate concentrated *in vacuo* to a volume of 20 ml. The presence of *desoxyribonucleases and nucleotidases* in fresh chick blood was shown by incubation of sodium desoxyribonucleate (Nutritional Biochemical Corp.) solution (10% in dilute alkali) with the chick blood, chromatographing and bioautographing. Descending chromatography according to the procedure of Winsten and Eigen(5) was used to separate the growth factors active for *Lactobacillus leichmannii* 4797. Ten to 15 μl of each of the blood concentrates, DNA preparations, liver extract, vit. B_{12} (100 mγ/ml) or thymidine (50 γ/ml) were spotted on ½ x 16 inch paper strips (Eaton Dikeman No. 613). Water saturated with n-butanol was used as the developing agent. After removal from the chromatographic jars, the strips were air dried and bioautographed according to the method of Winsten and Eigen(6). In order to facilitate greater diffusion of vit. B_{12}, paper strips were allowed to remain on the surface of the seeded agar during the 16-hour period of incubation.

Results and discussion. Autolyzed blood samples from the chick, turkey, turtle, sheep, pig and cow contained 6.0, 5.2, 5.8, 0.8, 1.4, and 1.4 mγ of vit. B_{12} activity per ml of blood respectively as determined with *L. leichmannii.* Such values are within the range of those reported by earlier workers(1,2).

Tracings of bioautographs of liver extracts, concentrates of autolyzed blood samples from the chick, turkey and turtle, crystalline vit. B_{12} and thymidine are illustrated in Fig. 1.

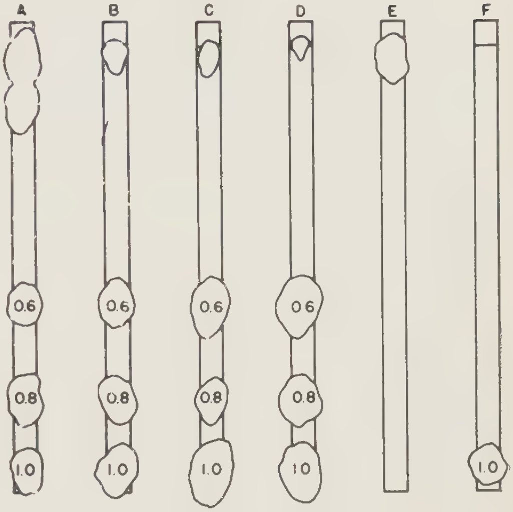

FIG. 1. Tracing of bioautographs. Relative rates* of active zones are given within the zone area. Strip A, 10 μl, 1:10 dilution of 15 U.S.P. unit liver extract (Lederle); Strip B, C, and D, 15 μl concentrates from autolyzed blood samples of chick, turkey, and turtle, respectively; Strip E, 10 μl sol. of crystalline vit. B_2 (100 mγ/ml); Strip F, 10 μl of thymidine sol. (50 γ/ml).

* Relative rate: (Distance from starting place to center of growth zone in question)/(Distance from starting place to center of growth zone for the fastest moving component)(7).

Liver extract solution (Strip A, Fig. 1) contains a stationary connected doublet zone (a part of which moved rather slowly) in addition to 3 more rapidly moving growth factors (6,7). Blood concentrates from chick, turkey and turtle (Strip B, C and D) each contained a growth factor for *L. leichmannii* 4797 which remained stationary as did vit. B_{12} (Strip E). Each of the concentrates also contained 3 additional growth factors which moved at the same relative rates (0.6, 0.8, and 1.0, Fig. 1) as did the 3 faster moving components of liver extract solution (Strip A, Fig. 1). The fastest moving growth factor in the liver extract and in 3 blood concentrates moved at the same relative rate as did thymidine (Strip F, Fig. 1). The growth factor in liver extract with the relative rate of 0.6 has been identified as hypoxanthine desoxyriboside(8). Blood concentrates from the chick, turkey, and turtle were also found to contain this component which moved at a relative rate of 0.6. An unidentified growth factor that moved with a relative rate of 0.8 was found to be present in liver extract and in these 3 blood concentrates. These data show that blood samples from species with nucleated erythrocytes contain, in addition to vit. B_{12}, 3 growth factors for *L. leichmannii* 4797. Such factors increase the apparent vit. B_{12} activity of these blood samples when assayed by a standard microbiological assay procedure with *L. leichmannii* as the test organism.

Blood samples from the cow, sheep and pig contain only one growth factor, which occupied the same stationary position on a bioautograph as did B_{12}. Attention is directed to the fact that blood from cow, sheep and pig contained only one growth factor for *L. leichmannii* 4797 while that from chick, turkey and turtle contained 4 growth factors for *L. leichmannii* (Fig. 1) even though the blood samples from all species were treated in an identical manner.

Alkali treatment of blood concentrates from the chick, turkey, and turtle destroyed the growth factor which occupied the same stationary position on a bioautograph as did vit. B_{12}. It has been shown that vit. B_{12} is destroyed by autoclaving in an alkaline medium (9, 10). Couch *et al.*(1) showed earlier that the *L. leichmannii* activity of blood samples from species with nucleated erythrocytes was decreased by autoclaving at pH 12 for 1/2 hour. Autoclaving blood samples from the chick, turkey and turtle in an alkaline medium did not change the relative position of the 3 faster moving growth factors on a bioautograph, which shows that these components are stable to such treatment.

Autoclaved fresh blood samples from the chick, turkey and turtle contained only one stationary growth factor which occupied the same relative position on a bioautograph as did vit. B_{12}. It is significant that the 3 faster moving components shown on Fig. 1 did not appear when the samples of chick, turkey and turtle blood were autoclaved immediately after collection. Autoclaving of fresh blood samples apparently destroyed blood enzymes (desoxyribonucleases, and nucleotidases), which were required for the release of the desoxyribosides from the desoxyribonucleic acids.

Chick blood solution or DNA solution when incubated alone were devoid of growth factors. Incubation of DNA solution with chick blood resulted in the appearance of the 3 faster moving growth factors. The fastest moving component moved at the same relative rate as did thymidine. It is obvious from these data that enzymes present in chick blood released 3 growth factors active for *L. leichmannii* 4797 from DNA.

Summary. Aqueous extracts of autolyzed blood samples from various species were chromatographed on paper strips and analyzed by bioautographic procedures. Under these conditions chick, turkey and turtle blood were found to contain 3 desoxyribosides in addition to vit. B_{12} while cattle, sheep and pig blood contained only B_{12} but not the desoxyribosides. Evidence is presented for the occurrence of desoxyribonuclease and nucleotidase in chick blood.

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Urinary Excretion of Free Alpha-Amino Acid Nitrogen by Normal Infants and Children. (19828)

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Quantitative data on the urinary excretion of alpha-amino nitrogen by adults have been published by Van Slyke, *et al.*(1), Albanese and Irby(2, 4), and by Thompson and Abdulnabi(3). Van Slyke, *et al.*, and Thompson and Abdulnabi used the gasometric-ninhydrin-CO_2 method devised by the former, while Albanese and Irby used their adaptation of the copper method of Pope and Stevens. There are, however, no reported values for the excretion of alpha-amino nitrogen by infants and children. This paper is concerned with the excretion of free alpha-amino acid nitrogen by normal infants and children, as determined by the gasometric-ninhydrin method of Van Slyke. The study was prompted by the absence of normal values in the literature, which were needed for comparison with values of alpha-amino nitrogen obtained from the urine of infants with diarrhea who were being given parenteral amino acid containing solutions.

Material and Methods. Twenty-four hour urine specimens were collected from 39 normal infants, premature infants and children. The urine was preserved with thymol and free alpha-amino acid nitrogen determinations were done the day the collection ended or the day after. All the individuals used in this study were well nourished, healthy infants and children. The children were the offspring of staff members of the Children's Medical Center or the Harvard Medical School, or ambulatory convalescent children residing at the Wellesley Convalescent Home. The infants were normal newborns or prematures from the Boston Lying-In Hospital, or the Infant's Hospital. All were on a normal diet which was in no way controlled. After the urine samples had been incubated with urease to destroy the urea, the free alpha-amino acid nitrogen of the urine was determined by the gasometric-ninhydrin-CO_2 method of Van Slyke, MacFadyen, and Hamilton(1). The results were expressed in mg alpha-amino acid nitrogen per day, and for convenience these were calculated in mg per lb of body weight per day. A total of 48 determinations were done on 39 individuals. Determinations were done more than once on several of the infants and prematures and there were 3 children upon whose urine determinations were made on two occasions.

Results. (See Table I).

Discussion. Table I indicates that the free alpha-amino acid nitrogen excreted in the urine of the children tested was about 1 mg per lb per day. There was no sex difference such as that found by Thompson and Abdulnabi(3) in their study of normal adults. The values found in this study were also appreciably lower than those reported in adults by them and by Albanese and Irby(2) and Albanese, *et al.*(4). The results obtained from infants and prematures, on the other hand, show an average daily excretion of about 3.9 mg per lb, and the difference between infants and children was statistically significant (p. $<.01$). This is in agreement with Barlow and McCance(5), as well as

TABLE I. Urinary Excretion of Free Alpha-Amino Acid Nitrogen.

			Mg lb/day			
Age group	No. subjects	Sex	Mean	Range	t	P
Children	34	Both	1.15	High 1.98		Male-Female
2-12½ yr	17	F	1.09			
	17	M	1.18	Low .71	.81	$>.4$
Infants						
1 day-3 mo.				High 14.60		Children-Infant
Prematures	14	Both	3.86		4.41	$<.01$
3 days-3 mo.				Low .82		

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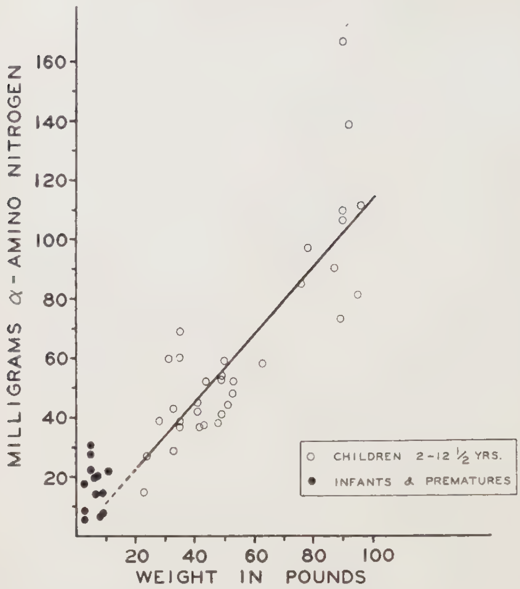

FIG. 1. Urinary excretion of free alpha-amino nitrogen by infants and children of various ages, expressed on a body weight basis.

Simon, and Goebel(6), who show that the proportion of total nitrogen excreted, attributable to alpha-amino acid nitrogen, was higher in the newborn and premature than in adults. Fig. 1 illustrates the results graphically and indicates that except for an occasional value, approximately 1 mg of alpha-amino acid nitrogen is excreted per pound of body weight, excluding the values for infants. The solid line represents the calculated mean for the determinations done on children. The broken line extension shows that among the values of free alpha-amino acid nitrogen for infants, all but 2 are above the calculated mean for children.

Summary. A series of 48 determinations of free alpha-amino acid nitrogen was done on the urine of 39 infants, premature infants, and pre-adolescent children. The children excreted approximately 1 mg per lb per day; the infants and prematures approximately 3.9 mg per lb per day. This difference is statistically significant. The values for children were lower than those reported for adults, and no sex difference was found.

The author is most grateful to Dr. Halvor A. Christensen in whose laboratory this work was done, for his direction and help.

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Distribution of C^{14}-Labeled Alloxan in the Tissues of the Rat and its Mode of Elimination.* (19829)

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A recent paper by Lee and Stetten(1) deals with the metabolism of alloxan-2-C^{14} and alloxan-1,3-N^{15} in rats. Following intraperitoneal administration of diabetogenic doses of these compounds, the isotopes were rapidly eliminated, chiefly by way of the urine. While the exact nature of the N^{15}-containing urinary excretory products was not determined, it was shown that they were neither ammonia nor urea, and probably not alloxan or alloxanic acid. The pancreas took up relatively small amounts of the labeled compounds.

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* This work was aided by grants from the Damon Runyon Memorial Fund and the National Institute of Neurological Diseases and Blindness.

TABLE I. Elimination of C^{14} in Urine and Expired Air Following Administration of Alloxan Labeled in Two Different Positions. Dosage was 11 mg/kilo in each case.

	Total radioactivity in % of administered dose					
	Expired CO_2		Urine			
Hr after inj.	Alloxan-5-C^{14}	Alloxan-2-C^{14}	Alloxan-5-C^{14}		Alloxan-2-C^{14}	
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
2	.19	.12			61†	
4½	.29	.25				
7½			68*		83	69
11			90	91		
22			93	95	91	92

* .24% of the C^{14} in this sample was due to urea.
† .27% " C^{14} " .

The present study is principally concerned with the rates of elimination of alloxan-5-C^{14} in the urine and expired air of rats, and with the distribution of this compound in the tissues of the body at various times following administration of the drug. In addition, some experiments were performed with alloxan-2-C^{14}, in order to compare its metabolism with that of the 5-labeled substance.

Methods. Male rats (250-275 g) of the Long-Evans strain were employed. Alloxan-5-C^{14} (0.86 μc/mg) and alloxan-2-C^{14} (1.85 μc/mg) were purchased from Tracerlab, Inc., Boston, on allocation from the U. S. Atomic Energy Commission. The labeled compounds were administered subcutaneously either at a "tracer" level of 11 mg/kilo body weight, or at the level of 110 mg/kilo, found to be diabetogenic in our laboratory. When the higher dosage was employed, the isotopic alloxan was diluted with 9 parts of ordinary alloxan monohydrate. At specified time intervals after injection, the animals were anesthetized with ether, and blood was withdrawn from the aorta. The tissues were quickly removed and frozen in dry ice. A sample of each tissue was homogenized with absolute ethanol. Aliquots of the resulting suspensions were pipetted upon aluminum discs. The blood was centrifuged, and 0.1 ml samples of plasma were spread uniformly on discs. After drying, the radioactivity of each sample was measured with a thin mica-window Geiger counter. The values were corrected for self-absorption. The kidney, plasma, and urine, all had relatively high C^{14} concentrations, and the samples were generally counted to within a probable error of 2 to 3%. The probable error was 5 to 8% with the other tissues studied. The values recorded in the tables and graphs are averages of duplicate determinations. These data were converted from counts per minute to % of total administered C^{14} per g fresh tissue, by reference to the specific radioactivity of the original alloxan-C^{14} preparations. In certain experiments, the rats were maintained in metabolic cages for the quantitative collection of urine. They were given food and water *ad libitum.* Suitable aliquots of the urine, collected at intervals, were plated for counting. In determining expired $C^{14}O_2$, an all-glass chamber was used, and the outgoing air was bubbled through a solution of CO_2-free NaOH. This solution was replaced by fresh NaOH at suitable intervals. Aliquots of each alkali solution were treated with excess $BaCl_2$, and the resulting $BaCO_3$ was washed with water and ethanol and plated for counting. Urea was precipitated from diluted urine samples with xanthydrol reagent, and the resulting dixanthyl urea was washed 3 times with methanol prior to plating for radioactivity measurements.

Results. Following the administration of either alloxan-5-C^{14} or alloxan-2-C^{14}, 90 to 95% of the C^{14} was accounted for in 22 hours in the urine (Table I). Lee and Stetten(1), employing alloxan at a level of approximately 200 mg/kilo, found the 2-C^{14} compound to be eliminated to the extent of only 67% in 2 days, while 57% of the alloxan-1,3-N^{15} was found in the urine in 2 days. These rates are somewhat lower than those observed in the present study.

Very low $C^{14}O_2$ concentrations were observed in analyzing the expired air. These accounted for less than 0.3% of the total

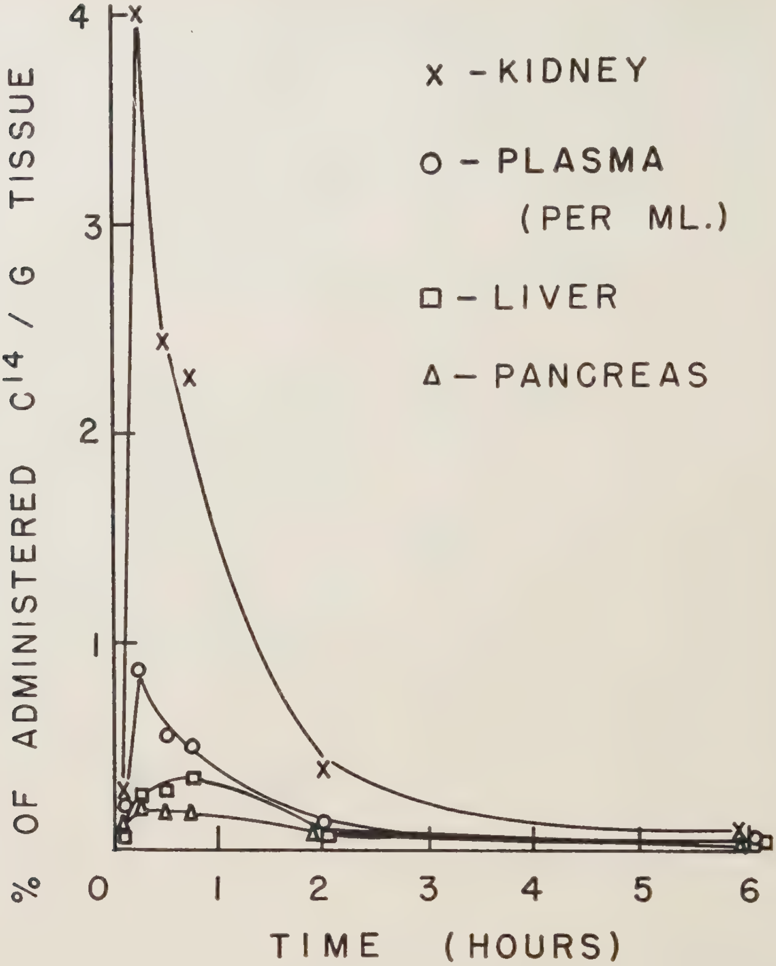

FIG. 1. Concentrations of C^{14} in tissues, following administration of 11 mg/kilo of alloxan-5-C^{14}. One animal was sacrificed at 5, 15, 30, and 45 min., and at 2 and 6 hr.

dose, after 4 1/2 hours, with either alloxan-2-C^{14} or alloxan-5-C^{14}.

Only a very minor proportion of the radioactivity in the urine was due to urea, with either type of C^{14}-alloxan (Table I). Lee and Stetten similarly found 0.8% of the N^{15} of alloxan-1,3-N^{15} to be due to urea. These virtually negligible activities in CO_2 and urea suggest that the alloxan molecule is not significantly catabolized in the animal body.

In exploratory starch column chromatograms by the Moore and Stein method(2) on urine from rats given alloxan-5-C^{14}, approximately 80% of the total C^{14} emerged in a sharp peak in the region of 20 ml of effluent. The nature of this component has not as yet been determined. It may be mentioned that both alloxan and alloxanic acid, likewise have peaks near 20 ml with the starch column. However, no positive conclusion can be drawn from these chromatograms, since the resolving power of starch for this class of substances has not been studied.

The relative concentrations of alloxan-5-C^{14} in 4 tissues after different periods of time are shown in Fig. 1. While the concentration of C^{14} in kidney appears to be several times greater than that in plasma, if the total quantity of kidney tissue (about 2 g) relative to that of blood plasma (approximately 10 ml) is taken into account, the quantities of C^{14} in the 2 cases are approximately equal at the

TABLE II. Radioactivity of Various Tissues Following Administration of Radioactive Alloxan.

	% of administered C^{14}/g fresh tissue			
	11 mg alloxan-5-C^{14}/kg		110 mg alloxan-5-C^{14}/kg†	
Tissue	15 min.	45 min.	15 min.	45 min.
Kidney	4.03	2.27	3.80	2.87
Plasma	.85*	.50*	.90*	.58*
Pancreas	.20	.15	.17	.17
Liver	.18	.40	.26	.50
Spleen	.21	.15	.16	.19
Adrenals	.14	.17	.13	.14
Salivary gland	.17	.17	.13	.16
Thyroid	.24	.21	.20	.21
Pituitary	.16	.19	.10	.14
Brain	.08	.04	.08	.07
Upper intestine	.12	.09	.11	.11
Skeletal muscle	.18	.08	.05	.05
	11 mg alloxan-2-C^{14}/kilo			
Kidney	3.75	2.65		
Plasma	.91*	.43*		
Pancreas	.18	.13		
Liver	.21	.24		

* Per ml plasma. † Animal was fasted 24 hr.

different time intervals. With both kidney and plasma the radioactivity was maximal at approximately 15 minutes. However, the C^{14} concentration in the liver reached a maximum 45 minutes after injection of the alloxan. It was of interest that only low concentrations of C^{14} were found in the pancreas. A similar observation has been made with alloxan-1,3-N^{15}(1).

A comparison of a variety of tissues with respect to alloxan-5-C^{14} uptake for two short intervals of time was made, with both high and low levels of the drug (Table II). The percentages of the C^{14} taken up were approximately equal with the 11 mg and 110 mg doses. The C^{14} concentration in the pancreas was approximately that in the other glands and tissues, except for kidney and plasma, which were higher, and brain, intestine, and skeletal muscle, which were somewhat lower. This finding relative to the pancreas seems of particular interest in view of the marked sensitivity of the beta-cells to injury by alloxan. The data for alloxan-2-C^{14} in Table II correlate reasonably well with those for alloxan-5-C^{14}, and this parallelism strengthens the view that the ureide and malonic acid portions of the alloxan molecule are not dissociated in the body. As with the 5-labeled compound, only relatively low C^{14} concentrations appeared in the pancreatic tissue.

In general the uptake of the two C^{14}-labeled alloxan preparations by the tissues is rather similar to the results with alloxan-1,3-N^{15}(1), particularly when the one-hour values with the latter compound are compared with the present 45 minute values. It should be pointed out that the values in Table II are for total C^{14}, and do not distinguish between protein and non-protein fractions. Actually, the experiments with alloxan-1,3-N^{15}(1) demonstrate that most of the isotope is in the protein-free filtrates of tissues.

The high radioactivity of the kidney tissue most probably reflects simply the passage of urine. Seligson and Seligson(3) have shown that alloxan is rapidly converted to alloxanic acid upon addition to blood plasma. The very high rate of elimination of the drug from the body tissues, and the negligible conversion to urea and carbon dioxide, make it seem improbable that alloxan is metabolized much beyond the alloxanic acid stage.

Summary. 1. Two alloxan preparations, labeled in the 2- and in the 5-position with C^{14}, were administered subcutaneously to rats. Both labeled compounds were rapidly excreted in the urine, to the extent of 90-95% in 22 hours. Negligible proportions of the C^{14} were found in urea. 2. With either tracer or diabetogenic doses, the radioactivity was highest in the kidneys and plasma, and reached a peak at approximately 15 minutes. The other tissues studied, including pancreas, had much lower C^{14} concentrations. 3. With either alloxan-2 or 5-C^{14}, very little radioactivity appeared as $C^{14}O_2$ in expired air. 4. It is concluded that the alloxan molecule does not undergo extensive catabolism in the rat.

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Influence of Nitrogen Mustards on Citric Acid Synthesis *in Vivo*.* (19830)

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During previous investigations on the influence of X-irradiation on intermediary carbohydrate metabolism we found(1) that citric acid synthesis is markedly depressed in hematopoietic tissues of irradiated rats. This effect was demonstrable through the use of the technic of sequential blocking of metabolic pathways developed by Potter(2) in which fluoroacetate is employed to inhibit the oxidation of citric acid *in vivo*(3-7). This procedure results in accumulation of citric acid in most tissues of normal rats in concentrations sufficient to permit quantitative measurements. The inability of certain tissues of irradiated rats to accumulate citrate following fluoroacetate treatment indicated that X-irradiation interferes with some reaction which normally leads to citrate formation. In contrast to an inhibition or no effect by X-ray on citrate formation in most rat tissues we observed that the livers of irradiated male rats accumulated large quantities of citric acid in the liver whereas normal male rats were unable to accumulate citric acid in the liver following fluoroacetate treatment(7-9). Recent studies in this laboratory(8) revealed that normal female rats and castrated or adrenalectomized male rats are able to accumulate citric acid in the liver following fluoroacetate treatment and thus resemble irradiated male rats. In addition, treatment of female rats with testosterone decreased citrate synthesis in the liver. These findings suggested that X-irradiation alters the metabolic pattern of the liver through interference with hormonal regulation of citrate synthesis.

The known similarities in the actions of nitrogen mustards and X-ray on mammalian tissues stimulated our interest in ascertaining whether the nitrogen mustards interfere with citrate synthesis in the same manner as X-ray. For this study various doses of the hydrochlorides of ethyl *bis*(2-chlorethyl)amine (HN1) and methyl *bis*(2-chlorethyl)amine (HN2) were administered to rats and the ability of various tissues to accumulate citric acid was measured following treatment of the animals with sodium fluoroacetate. These experiments demonstrated that the nitrogen mustards resemble X-irradiation in their effects on citrate formation in rat tissues *in vivo*.

Materials and Methods. Male Sprague-Dawley rats (175-225 g) were used for these experiments. Aqueous solutions of the hydrochlorides of HN1 and HN2 were administered intraperitoneally. In all the experiments rats were given 3.5 mg/kg of purified sodium fluoroacetate and sacrificed 3 hours later(2). Animals were sacrificed by decapitation and citric acid measurements were performed using the method of Natelson *et al.*(10) with the modifications recommended by Potter and Busch(7). The influence of HN2 on citrate formation and oxygen consumption *in vitro* was measured using the test system of Pardee and Potter(11) with various intermediates of the tricarboxylic acid cycle as substrates. Choline oxidase measurements were performed by the method of Williams *et al.*(12).

Results. Prior to studies on the influence of nitrogen mustards on citrate synthesis we measured the toxicity of HN1 and HN2 to rats under the conditions employed for this study. The approximate LD_{50} of the hydrochloride of HN1 was 0.75 mg/kg and the value for HN2 hydrochloride was 1.5 mg/kg when the compounds were given intraperitoneally to rats.

In previous studies we found(1) that X-irradiation exerts its most prominent inhibitory effect on citrate synthesis in spleen and thymus gland. Measurements of the influence of nitrogen mustards on citrate synthesis in spleen and thymus were, therefore, performed

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* This study was supported by funds provided under contract with the USAF School of Aviation Medicine, Randolph Field, Texas.

TABLE I. Effect of Ethyl *bis*(chlorethyl)amine (HN1) Hydrochloride on Citrate Accumulation in Spleen and Thymus of Fluoroacetate-Treated Rats.

mg/kg HN1 hydrochloride	μg citric acid/g fresh tissue Spleen	Thymus
Control	1425 (1245-1495)	1045 (965-1143)
1.5	892 (760-1150)	810 (745- 895)
2.0	895 (840-1160)	734 (610- 845)
2.5	744 (650- 830)	710 (545- 976)
5.0	420 (320- 688)	244 (130- 460)

in which both the dosage and time of sacrifice after HN1 and HN2 were varied. In all cases the animals were given 3.5 mg/kg of fluoroacetate 3 hours before they were sacrificed. The data in Table I show the effect of various doses of HN1 on the ability of spleen and thymus to accumulate citric acid following fluoroacetate treatment. The average values for 5 rats are presented together with the range of values for each group. The results of these measurements demonstrated that HN1 resembles X-irradiation in its inhibitory effect on citrate synthesis in spleen and thymus. However, in terms of the LD_{50} it was necessary to give higher doses of the nitrogen mustards than of X-ray to inhibit citrate synthesis in these tissues. Thus, 5 mg/kg of HN1 were required to produce an inhibitory effect comparable to that resulting from 800 r of X-ray. Whereas sublethal doses of X-ray (200 r) produced over 50% inhibition of citrate synthesis in hematopoietic tissues of rats only 38% and 22% inhibition of citrate synthesis occurred in spleen and thymus respectively in 48 hours after 1.5 mg/kg of HN1 which is twice the LD_{50} dose. Detailed studies on the influence of HN2 on citrate synthesis in spleen and thymus gland were also conducted in which several doses varying from 0.1 to 5 mg/kg of HN2 hydrochloride were given and groups of animals were sacrificed each day for 7 days following administration of the nitrogen mustard. After doses of 0.1 to 0.75 mg/kg of HN2 hydrochloride the amount of inhibition did not exceed 30% during the 7-day observation period. At 24 hours after 2 mg/kg of HN2 citrate accumulation was inhibited to the extent of 43% in spleen and 45% in thymus.

In the present investigation experiments were conducted to ascertain whether the nitrogen mustards resemble X-ray(1) in their effects on citrate accumulation in the livers of fluoroacetate-poisoned, male rats. The results of these experiments showed that the nitrogen mustards cause male rats to accumulate large quantities of citric acid in the liver following fluoroacetate treatment. In 24 hours after 1 mg/kg of HN1 hydrochloride the citrate content of the liver increased from an average for 6 rats of 64 μg/g to 354 μg/g of liver. With higher doses the effect was more pronounced as evidenced by an average of 729 μg of citric acid/g of liver in 24 hours after 2.5 mg/kg of the nitrogen mustard. More detailed studies were conducted with HN2 in which several sublethal doses were administered to male rats. Groups each containing 6 animals were treated with fluoroacetate and sacrificed at daily intervals for 7 days after each dose of HN2 and citric acid measurements were performed on the livers. An increase in citric acid accumulation in the liver was noted 24 hours after the administration of the doses employed in these experiments. The average value was generally highest about 4 days after administration of the nitrogen mustard. The data in Table II show the average value to-

TABLE II. Effect of Methyl *bis*(2-chlorethyl)amine Hydrochloride (HN2) on Citrate Accumulation in Livers of Fluoroacetate-Treated Rats.

mg/kg HN2 hydrochloride	μg citric acid/g fresh liver (4 days after HN2)
Control	64 (44- 71)
.1	269 (137- 430)
.25	338 (312- 364)
.5	449 (204- 812)
.75	642 (284-1025)
1	867 (331-1524)

TABLE III. Duration of Action of Methyl *bis*(2-chlorethyl)amine (HN2) Hydrochloride on Citrate Accumulation in Livers of Fluoroacetate-Treated Rats.

Days after 0.5 mg/kg HN2 hydrochloride	μg citric acid/g fresh liver
Control	64 (44- 71)
1	136 (101-182)
3	328 (255-453)
7	468 (198-872)
10	342 (230-434)
14	215 (123-418)
28	125 (61-226)

gether with the range for each group of animals at 4 days after poisoning. This experiment demonstrated that doses of HN2 far below the LD_{50} increase citric acid accumulation in the livers of fluoroacetate-treated, male rats.

The duration of the increase in citrate accumulation in the liver was measured by administering a single sublethal dose (0.5 mg/kg) of HN2 to rats and sacrificing groups containing 6 animals at intervals for a period of 4 weeks. The data in Table III show the persistence of the effect of HN2 on citrate accumulation in the livers of fluoroacetate-treated male rats. The average values together with the range of values for each group are presented in the table. These measurements demonstrated that the increase in citrate accumulation is evident at one day after administration of the nitrogen mustard and persists for several weeks. At 4 weeks after HN2 the values approached the normal range indicating that the change is eventually reversible following sublethal doses of the nitrogen mustards.

The experiments described above indicate that nitrogen mustards markedly alter carbohydrate metabolism in the liver. The nitrogen mustards are known to react with many biological compounds *in vitro*(13) but studies on the reactivity of these compounds with catalytic proteins have not yet provided an explanation for the toxic actions of low doses of the nitrogen mustards *in vivo*. In the present investigation the influence of HN2 on several oxidative reactions was measured using the test system of Pardee and Potter(11) with liver homogenates. Oxygen consumption and citrate formation was 50% inhibited by a final concentration of 2×10^{-3} M HN2 when pyruvate plus fumarate were used as substrates and the oxidation of acetate plus oxalacetate was 50% inhibited by 4×10^{-3} M HN2. The formation of acetoacetate from pyruvate in the presence of malonate(14) was 50% inhibited by 8×10^{-4} M HN2. The effect of HN2 on the oxidation of choline was also measured because choline oxidase has been reported(15) to be sensitive to the nitrogen mustards. The oxidation of choline by liver homogenates was inhibited 50% by 1×10^{-4} M HN2 when the test system of Williams *et al.*(12) was employed. These experiments demonstrated that relatively high doses of HN2 are required to affect these oxidative reactions *in vitro*.

To obtain a more direct evaluation of the action of HN2 on citrate synthesis and its effect on various oxidative reactions *in vivo*, experiments were performed on livers taken from rats which had been poisoned 2 days previously with 1 mg/kg of HN2. The ability of the livers of these animals to oxidize fumarate plus pyruvate and acetate plus oxalacetate was measured to obtain an indication of the effect of HN2 on enzymes involved in citrate synthesis. The formation of acetoacetate from pyruvate in the presence of malonate was measured to ascertain the influence of HN2 on pyruvate oxidation. Choline oxidase measurements were also performed on the livers of animals which had been treated with HN2. The results of these experiments are presented in Table IV in which the average values and the range of values for 4 animals are presented. These data show that 1 mg/kg of HN2 does not alter the ability of the liver to oxidize pyruvate plus fumarate or acetate plus oxalacetate. The oxidation of choline was likewise unaffected by this dose of the nitrogen mustard. The formation of acetoacetate from pyruvate was slightly increased. In contrast to the absence of effects by HN2 on enzymes involved in citrate formation by liver homogenates from HN2-poisoned animals the same dose of the nitrogen mustard caused a marked increase in the citrate content of the livers of fluoroacetate-treated rats. Thus, in two days after 1 mg/kg of HN2 the administration of fluoroacetate caused an increase in the citrate content of the liver of male rats to an average value of 889 μg/g as compared with 64 μg/g for normal male rats. The results of these experiments indicate that the increase in citrate formation in the livers of male rats after administration of HN2 occurs at dosages which have no direct effect on the enzymes involved in citrate synthesis.

Discussion. The results of these experiments have shown that nitrogen mustards produce alterations in citric acid accumula-

TABLE IV. Effect of Methyl *bis*(2-chlorethyl)amine Hydrochloride (HN2) *in vivo* on Ability of Liver to Oxidize Various Substrates. (Sacrificed 2 days after 1 mg/kg HN2).

	μM/50 mg liver/40 min.			
	Oxygen consumption		Citrate or acetoacetate formed	
Substrates	Control	HN2	Control	HN2
Pyruvate + fumarate	10 (9.3-10.8)	11.6 (7.7-14.5)	5.6 (4.8-6.4)	4.4 (3.9-5.4)
Acetate + oxalacetate	6 (5.5- 6.5)	7.7 (6 - 8.7)	2.2 (2.2-2.3)	2 (1.7-2.2)
Pyruvate + malonate	2.3 (1.9- 2.7)	3.3 (2.7- 4.2)	2.9 (2.3-3.5)	3.2 (3.1-3.4)
Choline	4.75 (4.2-5.3)	4.45 (4-5.1)		

tion in some tissues after treatment of rats with fluoroacetate according to the procedure developed by Potter(2). The nitrogen mustards thus resemble X-irradiation(1) qualitatively in their ability to alter citrate synthesis. In terms of LD_{50} values X-irradiation is considerably more effective than HN1 or HN2 in depressing citrate accumulation in spleen and thymus gland. Whereas doses below the LD_{50} of X-ray markedly inhibit citric acid formation in hematopoietic tissues it was necessary to administer lethal doses of the nitrogen mustards to obtain comparable effects. In contrast to the lesser effect of the nitrogen mustards than of X-ray on citrate synthesis in hematopoietic tissues the alteration in the metabolic pattern of the liver was more pronounced and consistent with the nitrogen mustards than with X-ray. However, in both cases male rats acquired the ability to accumulate large quantities of citrate following fluoroacetate treatment. That the increase in citrate accumulation following HN2 does not result from a direct action of the toxicant on reactions involved in the tricarboxylic acid cycle was suggested by the absence of significant changes in the ability of the livers from rats poisoned with HN2 to oxidize several intermediates of the citric acid cycle. The relatively high concentrations of the agents which were necessary to alter citrate formation and oxygen consumption *in vitro* also indicate that some indirect influence by the nitrogen mustards must be responsible for the metabolic changes observed by the use of the fluoroacetate technic(2). Our recent observations(8) that normal female rats and castrated or adrenalectomized male rats have the ability to accumulate citrate in the liver following fluoroacetate treatment suggest that a hormonal regulation of citrate synthesis is normally operative in the livers of male rats. The nitrogen mustards and X-ray may exert their influence on the liver through interference with the regulatory influence of hormones on citrate synthesis. The results of the present study have demonstrated a similarity in the action of X-ray and the nitrogen mustards on citrate synthesis in rat tissues. Elucidation of the exact mechanisms responsible for these biochemical changes requires further experimentation.

Summary. The influence of nitrogen mustards on citrate synthesis in rat tissues *in vivo* was studied using the fluoroacetate technic of Potter(2). The results of this study demonstrated that HN1 and HN2 produce alterations in citrate synthesis in certain tissues which are qualitatively similar to those produced by X-ray. Thus, a decrease in citrate synthesis was observed in spleen and thymus glands of nitrogen mustard-poisoned animals and a marked increase in citrate accumulation was observed in the livers of male rats. The increase in citrate accumulation in the liver was evident after doses far below the LD_{50} and persisted for several weeks following sublethal doses of the nitrogen mustards.

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Antidotal Efficacy of Vitamin B_{12a} (Hydroxo-Cobalamin) in Experimental Cyanide Poisoning. (19831)

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(Introduced by Hans Molitor.)

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It has been shown that vit. B_{12} (cyanocobalamin) contains one cyano group bound coordinatively to the cobalt atom, whereas in vit. B_{12a} (hydroxo-cobalamin) a hydroxo group is present instead of the cyano group(1). The addition of cyanide ions to a solution of vit. B_{12a} results in the formation of vit. B_{12} (2). That the cyano group is tightly bound within the coordination complex of vit. B_{12} is indicated by the observation that dose levels of vit. B_{12} up to 1600 mg/kg both intraperitoneally and intravenously are nontoxic to mice(3). Calculation reveals that a dose of 1600 mg/kg of vit. B_{12} contains the equivalent of about 32 mg/kg of cyanide ion, or about 8 times the LD_{100} dose.

The ease with which vit. B_{12a} reacts with cyanide to yield vit B_{12} and the apparent irreversibility of this reaction in animals suggested that vit. B_{12a} might influence beneficially the course of cyanide poisoning in a manner analogous to that by which B.A.L. counteracts arsenic poisoning(4). The present communication demonstrates that vit. B_{12a} is capable both of preventing and of reversing the toxic effects of cyanide in mice.

Material and Methods. Male mice of the Harpaul and Barckmann stocks, weighing 20-28 g, were employed in these experiments. Freshly prepared aqueous solutions of potassium cyanide were injected intraperitoneally, preceded or followed by the intravenous administration of vit. B_{12a} or physiologic saline. The volume of intravenously injected solutions was kept constant at 0.2 ml/20 g body weight. Exp. 1, 2, and 3 (Table I) were controlled by using a saline-treated group of mice at the beginning and again at the end of the test. In all other experiments alternate (pair-weighed) mice were injected with vit. B_{12a} or saline. The vit. B_{12a} was prepared by irradiation and aeration from crystalline vit. B_{12}(5) and was recrystallized from acetone.

Results. Data in Table I show that the prophylactic injection of vit. B_{12a} intravenously, approximately 20 seconds before the intraperitoneal administration of potassium cyanide, was effective in reducing the mortality due to the latter substance. Respiratory distress and convulsions were prevented completely or were minimized. Doses of 50 or 250 mg/kg of vit. B_{12a} gave adequate protection against doses of 5.5-8.0 mg/kg of potassium cyanide, whereas doses of 25 mg/kg of vit. B_{12a} or less did not. No prophylactic effect against potassium cyanide toxicity (7-8 mg/kg, intraperitoneally) could be shown for vit. B_{12} (cyano-cobalamin) given intravenously in doses of 50-250 mg/kg. The encouraging results obtained by pretreatment with vit. B_{12a} prompted a study designed to determine whether this compound would effect recovery after the toxic manifestations of potassium cyanide were evident.

Table II shows that an intravenous dose of 250 mg/kg of vit. B_{12a} administered within

TABLE I.
Protective Influence of Vitamin B_{12a} Against Lethal Action of Potassium Cyanide in Mice.

Exp.	Stock of mouse	No. of mice	IP dose KCN, mg/kg	IV dose B_{12a},* mg/kg	No. of deaths	% mortality
1	Harpaul	6	5.5	—	4	67
				50	1	17
				5	5	83
				—	4	67
2	”	6	7.2	—	2	33
				50	0	0
				5	4	67
				—	3	50
3	Barckmann	6	7.7	—	6	100
		6	7.7	250	0	0
		5	7.7	—	3	60
4	”	20	8	—	10	50
				(.5, 5, 25)†	12	60
5	”	12	8	—	10	83
				50	1	8
6	”	12	8	—	9	75
				50	1	8
Totals	Harpaul and Barckmann	42	5.5–8	50–250	3	7
		32	5.5–8	.5–25	21	66
		79	5.5–8	—	51	65

* B_{12a} inj. IV about 20 sec before KCN was administered IP.
† Data on 3 dose levels combined since all were ineffective.

TABLE II. Therapeutic Action of Vitamin B_{12a} in Mice* Poisoned with Potassium Cyanide. IP dose KCN, 10 mg/kg.

Exp.	No. of mice	IV dose B_{12a}, mg/kg	Interval between inj. of KCN and B_{12a}, (min.)	No. of deaths	% mortality
7	6	250	1	0	0
			2	0	0
			4	1†	17
	2	”	6	2	100
	3	”	8	3	
	23‡	—	—	23	
8	6	100	1	0	0
	6	”	2	2	33
	12‡	—	—	12	100

* Barckmann stock.
† Of 5 mice which recovered, 3 had shown cessation of respiration at the time vit. B_{12a} was inj.
‡ Individual mice received saline IV at same time intervals after KCN as their respective pair-weighed partners treated with vit. B_{12a}.

one or two minutes, or 100 mg/kg within one minute, after the intraperitoneal injection of an otherwise lethal dose of potassium cyanide, 10 mg/kg, was capable of preventing death. The severe respiratory distress and convulsions, present at the time of B_{12a} injection, disappeared immediately. Vit. B_{12a}, at 250 mg/kg or 100 mg/kg, given 4 or 2 minutes respectively after the injection of potassium cyanide brought about recovery of most mice, even in several animals exhibiting complete respiratory failure. The administration of vit. B_{12a} 6 or 8 minutes after potassium cyanide treatment was ineffectual.

To study further the antidotal action of vit. B_{12a} it was decided to evaluate the efficacy of this compound in mice "apparently dead" of cyanide poisoning, *i.e.*, mice showing no respiration nor responsiveness to external stimuli, such as handling. The majority of mice, thus treated, reacted dramatically to the injection of vit. B_{12a}. Respiration frequently

TABLE III. Recovery Effected by Vit. B_{12a} in Mice* "Apparently Dead" from Cyanide Poisoning. IP dose KCN, 10 mg/kg.

Exp.	No. of mice	IV dose B_{12a}, mg/kg	Time of "apparent death"† (sec after inj.) Mean	Range	Final No. deaths	% mortality
9	5	250	176	(150-215)	1	20
		—	155	(135-195)	5	100
10	6	250	159	(135-210)	5	83
		—	164	(145-240)	6	100
11	7	250	135	(120-155)	1	14
		—	191	(120-520)	7	100
12	3	250	147	(130-175)	0	0
		—	132	(120-150)	3	100
Totals	21	250	153	(120-215)	7	33
		—	166	(120-520)	21	100

* Barckmann stock.

† By "apparent death" is meant the state in which all respiratory movement has ceased and the animal does not react to external stimuli.

TABLE IV. Urinary Excretion Products of Mice Injected with Vit. B_{12a} (Hydroxo-Cobalamin) and KCN. 2.5 hr urine collection.

Excretion products	Amount in μg: Untreated controls (6)	Amount in μg: Mice inj. with B_{12a} & KCN (6)	As % of total amt of CN^- inj. (550 μg)
Total cobalamin	—	2950	
as CN^-	—	59	
Cyano-cobalamin (B_{12})	—	2625	
as CN^-	—	52.5	9.6
Free cyanide	—	3.7	.7
Thiocyanate	9.2	52.2	
as CN^-	4.1	23.4	3.5*

* 3.5% = 19.3 μg, difference between that found in urine of inj. (23.4 μg) and untreated (4.1 μg) mice.

returned even before the entire dose of vit. B_{12a} was injected and following completion of injection many of the mice were able immediately to walk. The reduction in mortality effected by vit. B_{12a} treatment is shown in Table III.

In an attempt to elucidate the mechanism by which vit. B_{12a} counteracts the toxic effects of cyanide, the following experiment was performed. A group of 6 mice was first injected intraperitoneally with 10 mg/kg of potassium cyanide and approximately one minute later was given 100 mg/kg of vit. B_{12a} intravenously. The animals were placed in a metabolism cage, with access to water, and urine was collected for a period of 2½ hours. The urine of 6 control mice was collected similarly. Results of analyses of the urines are presented in Table IV. The data show that, of a total of 550 mcg CN^- administered, 9.6% could be accounted for as cyano-cobalamin, 3.5% as thiocyanate and 0.7% as free cyanide.

Discussion. Cyanide ion exerts its acute toxic effect by combining with the metalloporphyrin containing enzyme systems concerned in tissue respiration. Most drugs used for the treatment of cyanide poisoning, such as sodium nitrite or methylene blue, act by virtue of converting hemoglobin to methemoglobin which in turn combines with cyanide to form cyanmethemoglobin. Subsequent treatment with thiosulfate brings about the formation of thiocyanate, a relatively nontoxic substance which is excreted, though somewhat slowly(6,7). The present experiments show that vit. B_{12a} is also effective in counteracting cyanide toxicity. It is evident that the comparatively large quantity of vit. B_{12} found in the urine of mice treated with vit. B_{12a} and potassium cyanide resulted from the reaction of these two compounds, but the precise site where this reaction took place is not known. Urinary excretion data for the first 2½ hours after injection indicate that some of the administered cyanide was detoxified by physiologic conversion into thiocyanate, but that a greater proportion of it was detoxified by combination with vit. B_{12a} to form vit. B_{12}.

The failure of vit. B_{12} to influence favorably the course of cyanide poisoning, despite its capability of binding 1 or 2 moles of cyanide in addition to the cyano group already present in its molecule(8) may be due in part to the lability of the resulting complex.

Summary and conclusions. 1. Vit. B_{12a} (hydroxo-cobalamin), but not vitamin B_{12} (cyano-cobalamin), has been found to be capable of preventing in mice the toxic symptoms and death due to cyanide administration. 2. When injected into mice exhibiting complete respiratory arrest and coma due to cyanide poisoning, vit. B_{12a} effected rapid recovery of most animals. 3. In mice injected with potassium cyanide followed by vit. B_{12a}, some of the cyanide appears in the urine as thiocyanate, but a greater percentage of the cyanide appears as vit. B_{12}, having formed this compound by reacting with the vit. B_{12a}.

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Crystalline Catalase, A Peroxidase. (19832)

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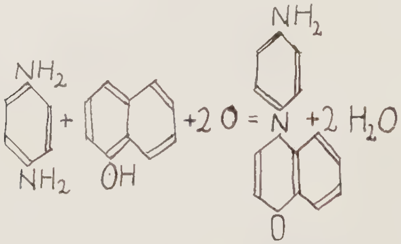
Catalase is generally classified as a non-oxidizing enzyme; its only function is said to be to destroy the toxic hydrogen peroxide. This classification was made because no other substrate has been found for this enzyme. Alkyl peroxides (which are not present in living organisms) are attacked only by concentrated catalase solutions(1). Keilin and Hartree(2) observed that a portion of the hydrogen peroxide, formed when certain oxidases aerobically oxidize their substrates, is utilized by catalase to oxidize ethyl alcohol to acetaldehyde. Oxidation by catalase and hydrogen peroxide was much slower. Theorell (3) and his school believe that catalases are a special group of peroxidases. Their deduction is based on studies concerning the mechanism of action of catalases and of peroxidases. No evidence is available, however, to indicate that catalase can oxidize any large or small molecule of biological importance, or any of the several types of the substances that are oxidized by peroxidases.

The experiments here described demonstrate a series of new functions of catalase. Since the reactions require the presence of hydrogen peroxide, they are peroxidatic in nature.

Experimental. Preparation of crystalline catalase. The crystalline cow liver catalase employed in these experiments was prepared according to the method of Tauber and Petit (4). The crystals which formed during dialysis were recrystallized by dissolving them in the least volume of 0.01 N sodium hydroxide. Without centrifuging, the solution was immediately adjusted to pH 5.8 by the addition of the calculated volume of 0.1 N acetic acid. A small quantity of insoluble matter was removed by centrifuging at room temperature. The clear supernatant was placed in a refrigerator at 4°. In about 3 hours the catalase began to crystallize. The crystals were dissolved in sodium chloride-phosphate buffer at pH 7.3(5). The activity of the crystals, as found by the method of von Euler and Josephson(6), was 34,000 Kat. F. The stock catalase solution was kept in a refrigerator. All reagents and solutions of the compounds used in these experiments were prepared daily. α-Naphthol and p-phenylenediamine were decolorized with activated charcoal. For comparison commercial (Worthington) crystalline catalase was also employed.

A. The Nadi reaction with catalase and hydrogen peroxide. 1 ml of freshly prepared α-naphthol solution (0.05 M in 95% ethyl alcohol), was placed in a test tube. One ml of freshly prepared p-phenylenediamine solution (0.05 M in distilled water), 4 ml of 0.05 phosphate buffer at pH 7.3, and 0.1 ml of 0.3 M solution of hydrogen peroxide were added. After mixing the solution was divided into 2 parts. To one-half 0.1 ml (10 μg) of catalase solution was added. Both tubes were kept for 5 minutes at room temperature. The tube containing the catalase turned purple (indophenol purple). Then 2 ml of 95% ethyl alcohol was added to each tube. After mixing the purple color in the catalase-containing tube became more brilliant. The control had a pink color. The reaction occurs in the following manner:



p-Phenylene-diamine α-Naphthol Indophenol purple

If dimethyl p-phenylenediamine is employed instead of p-phenylenediamine, indophenol blue is formed. A similar oxidation reaction is known to be catalyzed by the cytochrome oxidase-cytochrome c enzyme system. The name of the reaction, "Nadi," was derived from the compounds α-naphthol and p-phenylenediamine.

B. Oxidation of other large molecules by catalase and hydrogen peroxide. To 1 ml of each of p-aminobenzoic acid, sulfathiazole, adrenalin, ephedrine, and tyrosine solution, 1 ml of catechol (0.05 M) in 0.05 M pH 7.0 phosphate buffer, 0.2 ml of a 0.3 M hydrogen peroxide solution and 0.1 ml (2 μg) of catalase solution in phosphate buffer of pH 7.3 were added. The tubes were kept at 37°. After 5 minutes the appearance of colors was noted (Table I). The control tubes without catalase were either slightly colored or colorless. In the tyrosine experiment 10 μg of catalase were employed.

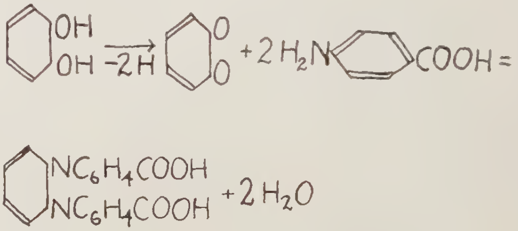
TABLE I. Peroxidation of Large Molecules by Catalase.

	Color after 5 min. at 37°	Changing gradually to
p-Aminobenzoic acid	Deep carmine	Red brown
Sulfathiazole	Rose red	" "
Adrenalin	Brick "	Deep red brown
Ephedrine sulfate	Pink	Lilac rose
L-Tyrosine	Rose red	Olive black

The compounds were dissolved in 0.05 M phosphate buffer of a final pH of 7.0, except the adrenalin which was in 0.05 M phosphate buffer of a final pH of 7.2, and the tyrosine solution which had a final pH of 8.3; the other substrates were adjusted to a final pH of 7.0 with sodium hydroxide. The concentrations were 0.005 M, except the ephedrine sulfate solution, which was 0.05 M.

Here, part of the oxidations probably occur in the following manner:

Catechol o-Quinone p-Aminobenzoic acid



Similar results were obtained with a commercial (Worthington) crystalline catalase preparation, and with crude horse radish peroxidase and hydrogen peroxide.

C. Peroxidation of phenols and amines by catalase and hydrogen peroxide. All 4 compounds of this group were dissolved in 0.1 M phosphate buffer of pH 7.3 and were of 0.05 molarity except the adrenalin solution which had a molarity of 0.005. Into 1 of each of 4 test tubes, 2 ml portions of each of the 5 compounds and 0.1 ml of 0.3 M hydrogen peroxide solution were added. The contents of each of the 5 tubes was divided into 2 parts.

To 1 part of each 0.1 ml catalase solution (10 micrograms) was added. Catechol gave an intense yellow color, pyrogallol a brown color, adrenalin a pink color, and p-phenylenediamine gave a light-reddish-purple color.

Discussion and summary. Our experiments show that dilute solutions of crystalline catalase, in the presence of hydrogen peroxide, can peroxidize a variety of compounds. α-Naphthol and p-phenylenediamine are oxidized to indophenol purple; p-aminobenzoic acid, sulfathiazole, adrenalin, ephedrine sulphate, and tyrosine, are coupled with catechol by oxidation to form colored compounds. Pyrogallol, catechol, adrenalin and p-phenylenediamine are oxidized by catalase and hydrogen peroxide. Related compounds (m-aminobenzoic acid, sulfadiazine) are also oxidized. A commercial crystalline catalase preparation, as well as crude horse radish peroxidase gave similar results. A catalase solution which had been boiled for 5 minutes did not give the described reactions.

The difference between catalases and peroxidases is: catalases can act as peroxidases but peroxidases can not act as catalases (decompose hydrogen peroxide without the presence of a second substrate). The present experiments are in full harmony with Theorell's theory which states that catalases are a special group of peroxidases.

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Combination Chemotherapy of Cancer with 8-Azaguanine and a Riboflavin Analog.* (19833)

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Quantitative biochemical differences between normal and tumor tissues have been reported by many investigators(1-5), and reveal, for the most part, lower metabolite concentrations in neoplastic tissue. Metabolite inhibition by an antimetabolite depends upon the concentration ratio of metabolite to antagonist. Thus, the function of a metabolite in low concentration in cancer cells may be blocked by an antagonist while only minimally depressing the metabolic activities of the higher metabolite level in normal cells. The possibility that the utilization of combinations of drugs against experimental neoplasms may result in simultaneous damage to different metabolic pathways, achieving greater tumor damage, has been reported by a number of investigators (6-9). For example, the vit. B_6 antagonist, desoxypyridoxine, was chosen for chemotherapeutic trial because it had been demonstrated that pyridoxine is in lower concentration in many solid tumors than in most normal tissues (1,3,4). Desoxypyridoxine was found to be a weak carcinostat against a mammary adenocarcinoma, and, in combination with a guanine antagonist (8-azaguanine), produced a carcinostatic effect not obtainable with either drug alone(6).

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† Fellow of the Dazian Foundation.

TABLE I. Effect of Flavotin Alone and in Combination with 8-Azaguanine on the 755 Carcinoma in C57 Mice.

Exp. No.	Group	Sex	Dose,* mg/kg	Mean tumor wt (mg)	No. of animals Dead/Total	% change in body wt
43A	Control	♂		2418	3/19	+12
	Flav.	♂	60	1687	1/18	+ 5
27B	Aza.	♂		954	0/20	+ 2
	Aza. + Flav.	♂	50	770	5/26	— 3
36A	Aza.	♂		969	0/20	0
	Aza. + Flav	♂	65	782	8/20	— 3
44	Control	♀		1795	1/20	+18
	Flav.	♀	60	1355	2/20	+ 9
49	Control	♀		1549	0/20	+12
	Flav.	♀	60	1160	1/20	+ 4
61	Control	♀		1831	0/20	+12
	Flav.	♀	60	1805	1/20	+ 8
29	Aza.	♀		736	0/20	+ 6
	Aza. + Flav.	♀	50	268	0/25	— 5
35B	Aza.	♀		1041	1/20	0
	Aza. + Flav.	♀	60	490	0/27	— 7
36B	Aza.	♀		699	0/20	+ 2
	Aza. + Flav.	♀	60	239	5/20	— 8
63	Aza.	♀		2496	2/20	+ 4
	Aza. + Flav.	♀	60	1310	0/20	— 6

* Refers to dose of Flavotin. 8-Azaguanine always administered at a dose of 50 mg/kg one hour after injection of Flavotin.

Intraperitoneal therapy was given depending upon the daily weight of the treated animals, injections usually being given daily. Therapy was begun upon well established tumors varying between experiments from 3 to 13 days old. The duration of tumor growth varied between experiments from 20 to 34 days.

Inasmuch as riboflavin has also been reported in lower concentration in many tumors than in most normal tissues(1,4), the effect of a riboflavin analog, 6-chloro-9-(1′-D-sorbityl)-isoalloxazine, or flavotin, was evaluated on a mouse breast cancer, alone and in combination with 8-azaguanine.

Methods. A transplantable mammary adenocarcinoma, the 755 tumor in C57 black mice was the neoplasm employed in this study. The C57 mice were maintained in plastic cages in an air-conditioned, constant-temperature room (74°F), and had access to Rockland pellets and water *ad libitum.* Mice 2 to 3 months old and weighing 18 to 25 g were inoculated with tumor fragments into the axillary region by the usual trocar method. As noted in the tables, treatment was begun upon well established tumors. At the termination of an experiment the tumors were removed and the wet weights were determined to the nearest milligram. Statistical analysis of the significance of the difference between two mean tumor weights (M_1 and M_2) was made and the results were considered beyond chance variation when $\frac{M_1 - M_2}{\sqrt{(\sigma_{M_1})^2 + (\sigma_{M_2})^2}} = 2.5$ or greater. The guanine analog, 8-azaguanine, was dissolved in 0.1 N NaOH as previously described(10). Flavotin, a water insoluble compound, was dissolved by heating in 100% propylene glycol, and diluted with distilled water to a 10% propylene glycol solution. Ampules, containing a solution of riboflavin-5-phosphate, were used as the source of the vitamin in the reversal experiments.

Results. The lack of inhibitory effect of flavotin, alone or in combination with 8-azaguanine, on the 755 carcinoma grown in male animals is summarized in Table I. In addition, Table I records the results of similar experiments with flavotin in females. It is to be noted that in no experiment did flavotin alone produce significant inhibition of tumor growth. However, increased carcinostasis was obtained when flavotin was administered one hour prior to the injection of 8-azaguanine to female mice bearing the 755 carcinoma as

TABLE II. Reversal of Carcinostatic Effect of Flavotin on the 755 Carcinoma in Female Mice by Riboflavin.

Exp. No.	Group	Dose,* mg/kg	Mean tumor wt (mg)	No. of animals Dead/Total	% change in body wt
60	Aza.	50	366	0/20	+1
	Aza. + Flav.	50 + 60	148	1/20	—3
	Aza. + Ribo.	50 + 100	270	2/20	—2
	Aza. + Flav. + Ribo.	50 + 60 + 100†	288	4/19	—1
68B	Aza.	50	564	0/20	—1
	Aza. + Flav.	50 + 60	254	0/20	—7
	Aza. + Ribo.	50 + 100	324	2/19	—7
	Aza. + Flav. + Ribo.	50 + 60 + 100†	523	6/20	—6
72	Aza.	50	578	0/19	+1
	Aza. + Flav.	50 + 60	272	1/19	—6
	Aza. + Ribo. + Flav.	50 + 100 + 60†	351	1/18	—6

* 8-Azaguanine injected one hour after administration of Flavotin or Riboflavin-5-phosphate.

† Flavotin injected 15 min. after administration of Riboflavin-5-phosphate; 8-Azaguanine injected one hour after administration of Flavotin.

Daily intraperitoneal therapy was begun upon well established tumors varying between experiments from 5 to 7 days old. The duration of tumor growth varied between experiments from 21 to 24 days.

compared to the tumor growth obtained when the 8-azaguanine was administered alone. 8-azaguanine's consistent inhibitory effect upon the growth of the 755 tumor has been previously recorded(6,10). The results of all experiments with this drug combination in female animals are statistically significant. Further analysis of the data reveals a minimal mortality rate and slight to moderate weight loss. The degree of weight loss present in the treated mice is far below a level which in itself would inhibit tumor growth.

The results of experiments designed to determine whether the carcinostatic action of flavotin is due to interference with riboflavin metabolism are presented in Table II. The data demonstrate that the addition of riboflavin-5-phosphate to the 8-azaguanine-flavotin drug combination completely nullified the increased carcinostatic effects produced by flavotin in two experiments (Exp. 60 and 68, Table II), and partially blocked flavotin's action in a third experiment (Exp. 72, Table II), converting its usual carcinostatic effect into a statistically insignificant result. The results achieved by the combination of 8-azaguanine and flavotin in the three experiments recorded in Table II are statistically significant.

Discussion. As recorded in the tables, there is a difference in the effect of the flavotin-8-azaguanine drug combination upon the tumor depending upon the sex of the host. In every experiment in which the 755 carcinoma was carried by female mice, significant inhibition of tumor growth over that produced by 8-azaguanine alone occurred, whereas none of the experiments on male mice revealed this increased carcinostatic effect.

This variation of response in the two sexes is not understood, but it is not without precedent. Taylor and Carmichael(11) reported that folic acid was more toxic to female than male mice of the DBA strain. Goldin *et al.* (12) reported a 2-fold sex difference in lethal toxicity to aminopterin in adult mice. Folic acid was found to augment the carcinostatic activity of 8-azaguanine upon the 755 carcinoma in male mice only(6). Female mice bearing a transplantable lymphosarcoma demonstrated greater regression of the tumor than males after treatment with compound E(13). Okey *et al.* noted sex differences in biotin deficiency symptoms in rats(14). There are many other examples of sex differences in metabolic response, but the mechanisms through which these variations are mediated have yet to be elucidated.

The structural similarity of flavotin to riboflavin, together with the positive results obtained in the reversal experiments, permit the conclusion that flavotin's mechanism of action in producing carcinostasis (when used in combination with 8-azaguanine) is due to interference with riboflavin metabolism. Moreover, the lack of carcinostatic activity when flavotin is used alone in female mice presents the interesting possibility of a specific relationship between 8-azaguanine and flavotin. Studies are in progress to clarify the suspected relationship. It is conceivable, for example, that flavotin prevents the degradation of 8-azaguanine into a molecule lacking carcinostatic activity. Folic acid was found to potentiate 8-azaguanine's carcinostatic activity against the 755 tumor in this manner(6,15).

The positive results obtained with the riboflavin analog, flavotin, together with the demonstration that flavotin acts as a riboflavin antagonist in this tumor-host system, furnish additional support for the hypothesis presented at the beginning of this report.

Summary. Flavotin, a riboflavin analog, had no effect on the growth of a transplantable mammary carcinoma. However, the combination of flavotin and 8-azaguanine was carcinostatic, but only in female mice. The sex difference in response is not understood. Reversal experiments utilizing riboflavin-5-phosphate reveal the mechanism of increased carcinostatic activity afforded by the drug combination to be due to interference with riboflavin metabolism.

The technical assistance of Misses P. Hayworth, A. Atti, and Mrs. E. Zanar is acknowledged.

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Surface Activities of Biotin and Biocytin. (19834)

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Evidence has been presented by numerous workers that biotin functions in a variety of systems concerned with a) decarboxylation of, or carbon dioxide fixation in, acetoacetate, aspartate, malate, oxalacetate or succinate, b) aspartic acid, serine, and threonine deamination, c) succinic acid dehydrogenation, and d) the synthesis of adenine, arginine, aspartic acid, bone carbonate, citric acid, citrulline, guanine, and unsaturated fatty acids. One explanation for the apparent multiplicity of functions of biotin is that the compound is involved in only one primary system whose failure in biotin deficiency manifests itself by a variety of secondary metabolic derangements. Williams and Williams have shown (1) that biotin is highly "surface active" as inferred from its remarkable effect in eliminating the diffusion current maximum encountered during the polarographic reduction of Cu^{++} at the dropping mercury electrode. If biotin is highly "surface active" and does indeed function in this way, for example, as the prosthetic group of a larger surface active molecule, or as a factor regulating cell permeability, then it is apparent that biotin-

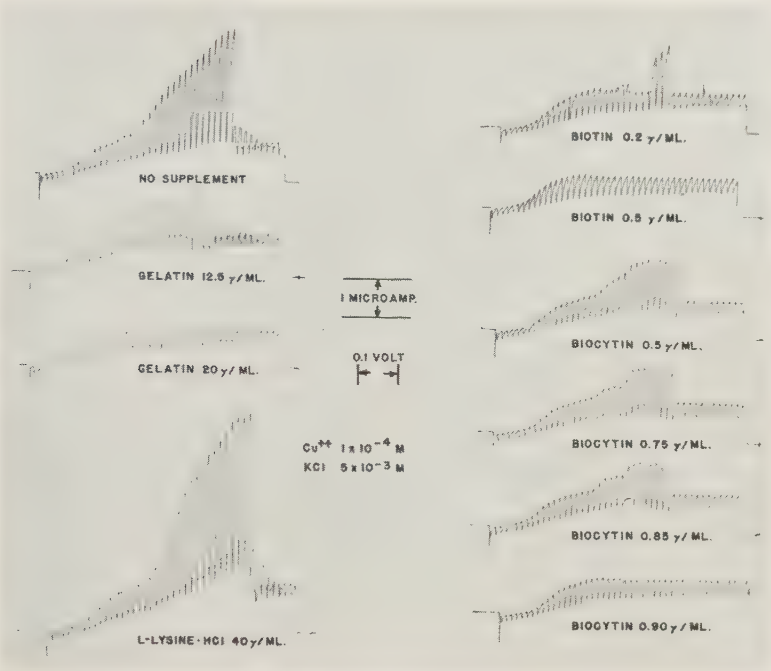

FIG. 1. The "surface activities" of biotin, biocytin, lysine, and gelatin as determined by concentrations required to eliminate the diffusion current maximum encountered during polarographic reduction of Cu^{++} at the dropping mercury electrode.

deficient cells would be expected to show a variety of impaired functions.

It is the purpose of this paper to present data a) confirming the activity of biotin in the system of Williams and Williams, and b) showing that biocytin, a "combined form" of biotin, recently identified as ε-N-biotinyl-L-lysine(2), also shows activity equal to that expected from the biotin content of the molecule.

Procedure. The data obtained were accumulated with a Leeds and Northrup Electrochemograph, Type E. The procedures followed in measuring the surface activities of the compounds studied were essentially those described by Williams and Williams and consisted of a determination of the minimal concentration of each compound required to just eliminate the diffusion current maximum encountered during the polarographic reduction of Cu^{++} at the dropping mercury electrode. After several trial runs, an aqueous solution of copper sulfate containing a copper ion concentration of 1.002×10^{-4} M was selected. As supporting electrolyte, to conduct the electrons through the solution, a concentration of 5×10^{-3} M KCl was found to be satisfactory. The range of potentials used in obtaining the polarograms was from 0 to –0.6 volt relative to the standard saturated calomel reference electrode. By trial and error, concentrations of biotin, biocytin, and gelatin were established, which, when each was dissolved in the KCl-$CuSO_4$ solution just described, would eliminate the potential maximum.

Results and discussion. The concentrations of the various compounds studied that were just sufficient to eliminate the diffusion current maximum under the conditions described (see Fig. 1) may be summarized as follows:

Biotin	.5 γ/ml
Biocytin	.9 γ/ml
Gelatin	20 γ/ml

These results indicate that biotin is the compound with the greatest activity studied. Biocytin is about 60% as active as biotin. This value agrees quite well with the biotin content of biocytin. L-Lysine is inactive in the present test.

An evaluation of the results summarized

above and an indication of the extent to which the effect observed is specific for biotin cannot be answered at this time because of paucity of similar data on a variety of biological compounds. The effects obtained would appear, however, to be of sufficient significance to warrant continued examination of the hypothesis that biotin may function through some "surface" mechanism.

Summary. The "surface activity" of biotin as measured by its effect in eliminating the diffusion current maximum encountered during the polarographic reduction of Cu^{++} at the dropping mercury electrode is confirmed. Biocytin (ϵ-N-biotinyl-L-lysine) is as active on an equimolar basis.

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Inhibitory Effect of Cobaltous Ions on Multiplication of Influenza Virus.* (19835)

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Interest in the action of cobalt on viruses was aroused by Burke's demonstration of the inhibition of growth and respiration of bacteria and animal tissues by cobaltous ions(1). It was subsequently found that cobalt salts inhibit the multiplication of influenza virus in embryonated eggs(2). Therapeutic trials conducted in our laboratory have yielded disappointing results, *i.e.* maximally tolerated doses of cobalt chloride administered orally and intranasally were of no value in the treatment of experimental influenzal infection in mice. Despite this fact, a study of the inhibitory action of cobalt was continued with the thought that some indirect information on the requirements for viral growth might be obtained. This report presents the results of further experiments dealing with the inhibitory action of cobalt on the viruses of influenza in embryonated eggs and the mechanisms of growth inhibition.

Methods. Three strains of influenza virus were used: namely, influenza A, PR8 and FM1, and influenza B, Lee. Groups of six to twelve 11-day-old chick embryos were inoculated with 0.1 ml of suitably diluted stock virus usually containing 100-500 ID_{50} by the allantoic route and returned to the 35 degree incubator. Inoculated embryos for each experimental series were removed from the incubator at the desired intervals and 0.4 ml aliquots of allantoic fluid were withdrawn from each of the eggs. The fluids were placed at 4°C as they were collected and maintained at that temperature until all eggs in a group were tapped, at which time they were assayed for their content of virus by hemagglutination tests employing the pattern endpoint (3) and, in some instances, by infectivity titration *in ovo*(4).

Results. When eggs were treated with 0.5 mg of cobaltous chloride or acetate at the time of virus inoculation, considerable inhibition of growth of PR8, FM1 and Lee viruses resulted. Results with the three strains of virus were essentially similar; in the experiments described the PR8 strain of influenza A was employed. The inhibition was most pronounced during the phase of rapid virus multiplication and persisted for a period of 32 hours after virus inoculation. Thus, in one series of 98 eggs treated with cobaltous chloride, only 11 failed to show at least a fourfold reduction in hemagglutination titer. Cobalt exerted a similar degree of inhibition when introduced at intervals varying from

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one hour before to 18 hours after inoculation of the virus; however, very little inhibition resulted when 24 hours elapsed between the inoculation of the virus and cobalt. The decrease in hemagglutinating titer of allantoic fluids was accompanied by a corresponding drop in infectivity. When eggs were treated with 0.5 mg of cobaltous chloride at the time of inoculation with virus, ID_{50} values of treated fluids 24 hours later were an average of three logs lower than those of the control fluids.

In order to determine the efficacy of the drug on larger and smaller inocula (100-500 ID_{50} having been used in previous experiments) the inoculum of virus was varied from 10 to 500000 ID_{50}. The results of this experiment indicated that the material was less effective when 1000 ID_{50} or more of virus were used. The agglutinating titers of these fluids showed only a 2-fold difference from those of the control eggs, as compared with the 5- to 10-fold difference evidenced in eggs receiving smaller inocula.

Treatment of infected eggs with 3 doses of 0.2 mg of cobaltous chloride at 0, 12 and 24 hours after inoculation resulted in a more prolonged inhibition than that observed with a single large dose. After 36 hours of incubation, there was still a 5-fold difference between the titers of the treated and control fluids, whereas the use of a single large dose resulted in only a 2-fold depression in hemagglutinating titer at this interval.

In order to determine whether cobalt salts had a direct inactivating effect on the virus, mixtures of the virus and cobaltous chloride were made, incubated *in vitro* for 2 to 8 hours, and tested for infectivity in embryonated eggs. Results of these experiments indicated that cobaltous ion, in concentrations approximating those existing in the chorioallantoic fluids of treated eggs, had very little virucidal effect *in vitro*.

Since histidine and cobalt combine (chelate) to form a cobalto-histidine complex(5) the action of histidine on the multiplication of influenza virus in eggs treated with cobalt salts was investigated. From the data presented in Table I, it is evident that the inhibitory effect on virus proliferation was reversed by the introduction of histidine into the system, the degree of reversal being proportional to the amount of histidine employed. Complete reversal was attained with a ratio of 2 mols of histidine per mol of cobaltous ion, the ratio existing in the cobalto-histidine complex. Cysteine, which chelates with cobaltous ion in a ratio of 3 to 1, respectively, was likewise active in overcoming the cobalt-induced inhibition when present in the ratio indicated. Sodium thioglycolate also forms a complex with cobalt but was much less effective in preventing the virustatic effect of cobalt, even in concentrations of 22 mols per mol of cobaltous ion.

TABLE I. Effect of Various Compounds on Multiplication of Virus in Cobalt-Treated Eggs.

Amino acids	mg	Yield of hemagglutinin (% of control) 24 hr
Alpha-alanine	4	19.6
Beta-alanine	4	12.3
Arginine	4	6.2
Aspartic acid	4	12.3
Cysteine	4	50
	8	96
Glutamic acid	4	6.2
Histidine	.5	28.5
	1	50
	2	57
	4	98.2
Phenylalanine	4	3.1
Tryptophan	4	12.3
Miscellaneous		
Sodium thioglycolate	5	38.5
	10	98
Imidazole	.5	13
Histamine	.5	12.3
Adenine	4	14.1
Thymine	4	16
Uracil	4	9.4

Inoculum: 100-500 ID_{50} of Influenza A (PR8) virus. Compounds listed were administered simultaneously with 0.5 mg $CoCl_2$ at time of virus inoculation.

In view of the observation by Broquist and Snell(6) that purine bases are converted to histidine in the embryonated egg, adenine, uracil and thymine were tested for their ability to counteract the cobalt effect. All proved to be inactive. The major components of the histidine molecule, imidazole and alpha-alanine, when tested individually and in combination, were also found to be devoid of activity.

Discussion. Knight has shown that highly purified suspensions of influenza virus contain histidine(7). From this it could be argued that cobalt inhibits virus growth by depriving the virus of histidine, an essential nutrient. However, cysteine which has not been shown to be a constituent of influenza virus and sodium thioglycolate which should be inert nutritionally also counteract the virustatic action of cobalt. Both the imidazole group of histidine and the sulfhydryl (–SH) group of cysteine or thioglycollic acid with which cobaltous ions chelate are also important chemically active groups in larger protein and nucleic acid molecules. Recent work by Burney and Golub(8) has suggested that –SH groups are present in the elementary bodies of psittacosis. These authors have demonstrated that p-chloromercuribenzoic acid completely inactivates psittacosis virus and that glutathione or cysteine reactivates the virus. In analogous experiments Klein *et al.*(9) have restored influenza virus activity with BAL and thioglycolate following inactivation by mercuric chloride. The influenza virus-cobalt-cysteine and virus-cobalt-thioglycolate relationships described above may involve –SH groups as well as imidazole groups in compounds necessary for viral growth. The compounds might be constituents of the virus particle or cellular enzymes of the host. With a sufficient excess of either the –SH bearing compounds, cysteine and thioglycolate or of histidine with its imidazole group, cobaltous ions would be removed from complexes previously formed through both types of linkage; *i.e.* chelation between cobaltous ions and imidazole groups or between cobaltous ions and sulfhydryl groups.

Summary. The growth of the virus of influenza in embryonated eggs is inhibited by cobaltous ion. This inhibition is overcome by the subsequent addition of histidine, cysteine and sodium thioglycolate.

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Indwelling Pulmonary Arterial and Venous Catheters in the Dog.* (19836)

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In order to obtain pressure measurements or blood samples from the pulmonary vessels of unanesthetized animals over a long period of time, investigators have usually resorted to externalizing the blood vessels to the chest wall by various types of cannulas. This requires major surgery. The following method describes a relatively simple procedure for placing and securing polyethylene tubes in the pulmonary artery or vein without resort to major surgery. They are introduced through the external jugular vein and common carotid artery, respectively. Catheters in the pulmonary artery have been retained in dogs up to two months. Such animals seem to keep in perfect health.

Placing the catheter into the pulmonary artery. Polyethelene tubes having an inside diameter of 1.27 mm (Clay-Adams cat. No.

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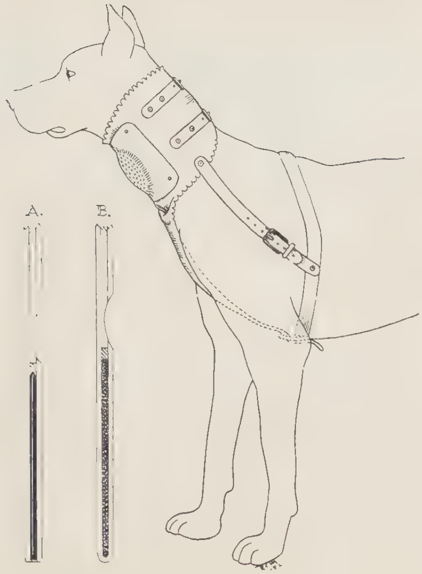

FIG. 1. A collar designed to protect the free ends of the indwelling tubes leading to the pulmonary artery and vein. A perforated metal plate bulges outward over the area of the incision. For fluoroscopic visualization the polyethelene tips were provided either with a nail (A) or mercury (B). (See text.)

PE 90) were cut to 100 cm lengths. In order to visualize the tip under the fluoroscope, our original method was to push a small, tight fitting nail into the end of the tube, after first removing its point and head. The metal piece was then sealed in place with glyptal and a smooth edged hole cut in the tube just behind the nail. More recently we have sealed off one end by heat and filled the tube with mercury for a distance of about two centimeters. A small hole is cut just above the level of the mercury which is sealed in place with glyptal. This latter method has the advantage of supplying a flexible catheter tip which is less likely to cause tissue damage. Longitudinal sections of each type of catheter tip are shown in Fig. 1.

The dogs were anesthetized with nembutal, and their necks shaved and cleaned. A section of jugular vein was exposed through a small incision, tied off distally, and the tube inserted through a small cut in the vein. A slow saline drip was started. With the dog lying on its side, the catheter tip was visualized under the fluoroscope and advanced into the right ventricle. In order to move the tip beyond this point, it was necessary to remove the saline drip and insert a stiff, flexible wire to within about 2 cm of the opening. With this extra support the tip of the catheter could now be moved along the inside curvature of the ventricle towards the pulmonary artery. This was most readily accomplished by advancing the tube a few centimeters, withdrawing the wire a few centimeters, advancing the tube, etc., until the tip was headed towards the artery. The wire was then withdrawn and the tip brought to the desired position in the artery, usually the diaphragmatic branch. After the saline drip had been started again, the loose ends of a tight ligature securing the catheter to the vein were sewn through the skin and tied. In order to permit drainage, no other stitching was done. The tube was then filled with heparin solution (50 mg/ml), and plugged, folded in loops, and taped to the neck with thin strips of adhesive tape. 600000 units of procain-penicillin were injected intramuscularly. A collar was designed to protect the free end of the catheter and yet allow free access of air to the incision. It consists of a wide piece of leather which covers the whole neck. A section of perforated sheet metal bulges outward over the area of the incision, allowing air to reach the wound. The collar is fastened with buckles and straps. To prevent twisting of the collar, a leather strap is fastened around the body just behind the forelegs and three straps connect it to the collar, 2 near the shoulder and one between the legs. The collar, as it appears on the dog, is illustrated in Fig. 1.

Placement of catheter into the pulmonary vein. The polyethelene tubes were prepared similar to those described above except that they are about 200 cm in length. A section of the common carotid artery was exposed through a small incision and tied off distally. A No. 10 radio-opaque cardiac catheter was inserted into the artery with a pressurized saline drip. After the catheter had been advanced in a retrograde manner into the low pressure system of the pulmonary vein, the saline drip was discontinued and the polyethelene tube threaded through the catheter until its tip was visible under the fluoroscope. By careful manipulation the regular cardiac catheter could be withdrawn leaving the polyethelene tube in place. Two fine wire ligatures were tied around the carotid artery to secure the tubing and stitched through either side of the incision in such a way as to externalize the cut end of the artery.

Results. Wound healing was found to occur within about 10 days provided penicillin was infiltrated in the incision during the operation. Seven dogs were prepared with indwelling catheters in the pulmonary artery and lived in apparent health and good spirits. Three of these animals retained these tubes for 41, 50 and 56 days, respectively. No clotting occurred in the tubes provided the heparin was changed at least once or twice a week. Blood samples could be withdrawn at any time and the pressures measured. At various times after successful healing in of the pulmonary arterial tubes, the catheterizations of the pulmonary veins were made. Contrary to the successful results on the pulmonary arterial side apparently considerable trauma is caused by the pulmonary venous catheters. In spite of all possible therapeutic measures wound healing was slow. The dogs lost their spirit and usually succumbed within 10 days unless the catheter was withdrawn. Autopsies revealed interlacing antemortem clots, raised hard plaques lining the vessel walls and inflammation of heart valves. The evidence strongly indicates that death was a consequence of the placing of the pulmonary venous catheter.

Summary. A method is described for placing permanent polyethelene catheters into the pulmonary artery and vein of dogs through the jugular vein and common carotid artery, respectively. The pulmonary artery catheter seems to cause little trouble to the animals and has been kept in place up to 56 days for sampling blood and measuring pressures. The pulmonary vein catheters, however, cause considerable trauma and animals succumb within 10 days after placement of the tube.

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Further Investigation of Effect of Colloidal Materials upon the Shwartzman Phenomenon.* (19837)

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When the Shwartzman phenomenon is elicited at two-day intervals in rabbits, successive skin lesions rapidly diminish in intensity and animals soon become completely non-reactive (1,2). The production of hemorrhagic skin lesions is not necessary for the development of resistance, repeated parenteral administration of bacterial materials being effective in inducing the state of non-reactivity(2,3). This type of acquired resistance to the Shwartzman reaction resembles closely the tolerance to the pyrogenic action of bacterial endotoxins which develops in the course of repeated injections of these materials in animals or humans(4), one similarity being that both types of resistance are abolished promptly by injection of thorotrast or trypan blue(1,5). This has been interpreted as indicating a role of the fixed phagocytes of the "reticulo-endothelial system" in the removal of bacterial toxins from the blood stream(1).

In the original studies of Beeson on the Shwartzman phenomenon, thorotrast was administered 16 hours before the intravenous provocative injection of bacterial toxin(1). The present report describes experiments designed to determine the effect of giving thorotrast at varying times before the provocative injection in resistant animals as well as observations on the effect of administration of a colloidal iodide preparation upon non-reactivity to the Shwartzman reaction.

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TABLE I. Effect of Administration of 9 ml of Thorotrast at Varying Times Before I.V. Provocative Injection of *S. marcescens* Filtrate in Shwartzman-Resistant Rabbits.

No. animals	Interval before provocative injection	Results Pos.	Results Neg.
4	30 min.	3	1
5	2 hr	5	0
2	4	2	0
2	8	2	0
2	16	2	0
4	24	4	0

TABLE II. Effect of Administration of 3 ml of "Angiopac" at Varying Times Before I.V. Provocative Injection of *S. marcescens* Filtrate in Shwartzman-Resistant Rabbits.

No. animals	Interval before provocative injection	Results Pos.	Results Neg.
5	30 min.	3	2
6	2 hr	5	1
2	4	2	
2	8	2	
3	16	3	
2	24	2	

Experimental. Material used to elicit the Shwartzman reaction was a filtrate of agar-washings from a culture of *Serratia marcescens.* The dosage employed (0.5 ml of 1:10 dilution intradermally and 1.0 ml of 1:25 dilution intravenously 24 hours later) produced typical hemorrhagic lesions in about 90% of normal animals. White male rabbits of mixed breed weighing 2000-3000 g were used. Hair was removed from the abdomen with electric clippers and intradermal injections were given in alternate sides of the abdomen beginning in the right upper quadrant and moving the sites of successive injections posteriorly. The general plan of experiments was to induce resistance in animals by eliciting the Shwartzman reaction on alternate days. After at least two negative results, animals were again prepared for the reaction by intradermal injection of filtrate and, at varying intervals before the provocative injection 24 hours later, one of the colloidal materials under study was injected. The two colloids used were thorotrast, a suspension of approximately 25% thorium dioxide (Heyden Co.), and Angiopac (lot No. 115 D.G., S.A. Union Chimique, Belge, N. V. Brussels), a colloidal organic iodide preparation which has been used in Europe for radiographic demonstration of the liver and spleen.

Results. The administration of a single injection of 9 ml of thorotrast immediately following skin preparation or at any time up to 30 minutes prior to the provocative injection of filtrate regularly abolished resistance to the Shwartzman phenomenon. Animals thus treated developed large areas of hemorrhagic necrosis at the prepared skin area. Table I presents a summary of results obtained with thorotrast.

Resistant animals given a single injection of 3.0 ml of Angiopac immediately following intradermal preparation or at intervals up to 30 minutes before the provocative injection showed typical Shwartzman lesions at the prepared skin areas, indicating that this colloidal material is also effective in abolishing resistance to the Shwartzman phenomenon. Table II summarizes findings in animals given Angiopac.

An attempt was made to determine the effect of giving thorotrast more than 24 hours before the intravenous provocative dose of bacterial filtrate. Under these circumstances, animals had already received thorotrast when the intradermal preparatory dose of filtrate was injected 24 hours prior to the provocative injection. Difficulty was experienced in interpreting results of such experiments because it was noted that thorotrast treatment prior to skin preparation often resulted in the appearance of significant amounts of hemorrhage 12 to 24 hours after the preparatory injection. Further study of this apparent enhancement of the local injurious effect of *S. marcescens* filtrate was carried out in normal rabbits. In two experiments, 32 animals were given 0.5 ml of 1:10 dilution of filtrate intracutaneously and the resulting lesions were recorded by tracing their extent on x-ray film with colored pencils at intervals up to 48 hours. In no instance was gross skin hemorrhage observed. Sixteen animals were then given 9.0 ml thorotrast intravenously, the remainder serving as controls. Twenty-four hours after thorotrast injection, all animals were again given 0.5 ml of 1:10 dilution of filtrate intradermally, the progress of the inflammatory reaction at the injection site being followed as before. Although hemorrhage was observed in none of the control animals, 11 of the 16 given thorotrast developed gross hemorrhage at the local site within 24 hours. In all thorotrast-treated animals, the edema and redness of the prepared skin area were much more extensive than that in the control group or in the same animals before thorotrast injection. In 8 thorotrast-treated animals, the intradermal injection of *S. marcescens* filtrate was repeated after a 21-day rest period. The resulting skin reactions resembled those observed before thorotrast injection; in no instance was skin hemorrhage seen.

Finally, the observation of Beeson(1) that thorotrast injection potentiates the lethal effect of intravenous injection of Shwartzman toxin was amply confirmed. Of 35 animals which had survived repeated injections of 1.0 ml of 1:25 dilution of *S. marcescens* filtrate, 21 died within 24 hours following injection of the same amount of filtrate after thorotrast treatment. Of 19 animals given Angiopac, 7 died wihin 24 hours after injection of *S. marcescens* filtrate intravenously.

Discussion. The results of the experiments reported here indicate that the injection of colloidal materials profoundly decreases resistance to the Shwartzman reaction within 30 minutes and that this interference is effective for at least 24 hours. Furthermore, both the local injurious and lethal properties of bacterial toxin are strikingly enhanced by these materials. Sickles(6) reported augmentation of the Shwartzman reaction by intravenous injection of India ink 20 minutes before the provocative dose of bacterial toxin.

Particulate substances have long been known to decrease resistance to experimental infections(7,8). Martin and his co-workers (9) have shown that thorotrast interferes with splanchnic removal of bacteria in animals with bacteremia produced by constant infusion of a suspension of organisms and have suggested this as a possible reason for the adverse effect of colloids on infections. Beeson(5) found that the development of tolerance to the pyrogenic effect of bacterial endotoxins in rabbits is accompanied by more rapid clearing of these materials from the blood stream after injection and that thorotrast, which abolishes tolerance, also interferes with the removal of pyrogen from the circulation. The fact that colloids localize in the macrophages of the reticulo-endothelial system after injection and that their effect upon infections and upon resistance to bacterial toxins seems to be related to interference with clearing the blood stream, makes "reticulo-endothelial blockade" a likely and tempting explanation of the effect of colloids upon resistance. However, the controversies surrounding the concept of true blockade of the macrophage system(7,10) and the absence of direct evidence bearing on the matter due to lack of a specific test of reticulo-endothelial function emphasizes a need for further investigation of this problem. It should be mentioned that pyrogen-tolerant rabbits evidence no increase in ability to clear bacteria from the splanchnic circulation(11).

Summary. The injection of thorotrast or Angiopac, a colloidal organic iodide preparation, abolishes resistance to the Shwartzman reaction within 30 minutes and for at least 24 hours. Treatment with these materials enhances strikingly the lethal effect of bacterial toxin and animals given thorotrast manifest increased susceptibility to the local injurious effect of intradermally injected toxin.

Mrs. Frances Brown and Mrs. Anne McManus gave technical assistance in this work.

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Utilization of Pteroylglutamic Acid Antagonists for Growth by a Strain of *Streptococcus faecalis*.[*] (19838)

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The toxicity of certain pteroylglutamic acid (PGA) antagonists has been demonstrated in a wide variety of species including microorganisms, rats, mice, the chick embryo and man. In microorganisms, Jukes, *et al.*(1) observed the inhibitory effects of several of the PGA antagonists for *Streptococcus faecalis* (ATCC No. 8043) and it was also noted that 4-amino-PGA (aminopterin) was toxic for the growth of *Escherichia coli*(2). The inhibition of growth of *Lactobacillus casei* by PGA antagonists was studied by Hutchings, *et al.*(3) and as with *S. faecalis* the toxicity was reversed under limited conditions by PGA. Sauberlich and Baumann(4) found that *Leuconostoc citrovorum* (ATCC No. 8081) was inhibited by aminopterin and this toxicity could be reversed by the citrovorum factor (CF). Studies with rat liver slices have revealed that aminopterin was effective in blocking the enzymatic conversion of PGA to CF (5). Kidder, Dewey and Parks(6,7) and Tittler and Belsky(8) observed that one of the PGA antagonists, aminopterin, could replace PGA in the nutrition of *Tetrahymena geleii*. Kidder *et al.*(6) further reported that 10-methyl-PGA (methopterin) could also substitute for PGA but not as effectively as aminopterin. 4-amino-10-methyl-PGA (amethopterin) on the other hand, could not substitute for PGA and was an inhibitor even in the presence of PGA. The toxicity of 4-amino-9-methyl-PGA (aninopterin) and 4-amino-9, 10-dimethyl-PGA (adenopterin) could be reversed by aminopterin and methopterin if concentrations equivalent to PGA were used.

A strain of *S. faecalis* resistant to 4-amino-10-methyl-PGA (amethopterin) has been shown to be capable of utilizing certain PGA antagonists for growth in lieu of PGA(9,10). This strain of *S. faecalis* to which amethopterin resistance was induced, hereinafter designated as *S. faecalis*/A, has been the subject of the present study in which a number of PGA analogs have been investigated for their effect upon the growth of this organism.

Methods. The microorganisms used in this study were *S. faecalis* (ATCC No. 8043) and *S. faecalis*/A. Both organisms were maintained in agar stabs and were transferred once a month to fresh media. *S. faecalis* was carried on Difco micro inoculum agar and *S. faecalis* /A was carried on Difco folic acid assay broth plus amethopterin (20 γ/ml), PGA (10 mγ/ml) and agar (1.5%). The technic employed for all growth experiments was that customarily used in microbiological vitamin assays.

Results. The ability of *S. faecalis*/A to utilize certain PGA antagonists to meet its requirement for PGA has been reported(9,10). When this resistant strain was compared with the parent strain it was found that the PGA requirement for half maximum growth (HMG) was appreciably decreased, from .3 to .1 mγ/ml. This was also the case with 10-formyl-PGA (.1 compared with .5 mγ/ml), pteroyl-glutamyl-glutamyl-glutamic acid (teropterin) (25 compared with 50 mγ/ml) and even a

TABLE I. Utilization of PGA Antagonists for Growth by *S. faecalis*/A.

PGA analog	mγ/ml	
	HMG	Growth range
"9-methyl-"*	3000	150 - 50000
10-methyl-	10	.5 ->500000
4-amino-	.25	.005->500000
-10-methyl-	25	5 - 100000
Pteroyl-aspartic-acid (PAA)	5	.05 - 250000
4-amino-PAA	2.5	.5 - 500000
-pteroyl-alanine	50	.01 - 25000
-teropterin	100	50 ->500000
10-methyl-pteroic acid	2.5	.25 - 500

* This preparation contained 2 components one of which did and one of which did not support growth of *S. faecalis*/A (see text).

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[†] Research Fellow of the National Cancer Institute.

TABLE II. Inhibition* of *S. faecalis* (8043) and *S. faecalis*/A at Two Levels of PGA and CF.

PGA analog	*S. faecalis* (8043)				*S. faecalis*/A			
	PGA		CF		PGA		CF	
	10 mγ	100 mγ	10 mγ	100 mγ	10 mγ	100 mγ	10 mγ	100 mγ
"9-methyl-"	500	200	30	3000	9000	>10000	>100000	>10000
10-methyl-	.3	.3	.1	3	6000	600	6000	600
9-10-dimethyl-	1	1	3	3	800	800	80	20
4-amino-	.2	.2	.3	1	—	—	—	—
-9-methyl-	.3	.03	1	2	7500	1500	4500	1000
-10-methyl-	.1	.03	.5	.5	60000	8000	10000	20000
-9-10-di-methyl	.1	.01	.05	.08	2000	200	300	200

* Inhibition: Ratio of antagonist to PGA or CF for H.M.G.

greater decrease was noted with pteroic acid (.1 compared with 50 mγ/ml). The CF (leucovorin) requirement was not altered (.5 compared with .4 mγ/ml).

Studies with other PGA analogs have led to the observation that certain of the PGA antagonists may also be substituted for PGA in a PGA deficient medium. The data in Table I give the HMG requirements and the growth range of *S. faecalis*/A on a series of PGA antagonists. The fact that 4-aminoteropterin did not support growth at a low concentration was to be expected since the unsubstituted triglutamate was not a particularly effective substitute for PGA.

The high concentration of "9-methyl-PGA" required for HMG and the rather narrow range over which the compound supported growth suggested the possibility that the compound was impure. By means of paper strip chromatography using an acetic acid-aqueous butanol system followed by bioautography of the chromatogram on a medium containing 1 mγ/ml of PGA and inoculated with *S. faecalis* (8043) two zones of inhibition were observed. One of the components had an Rf value of 0.63-0.68 which was comparable to other 9-methyl analogs, and a second component had an Rf value of 0.21-0.28. The identity of the slower moving component is not known. In another similar experiment it has been shown that the slower moving compound was capable of supporting the growth of *S. faecalis*/A, but that the faster moving compound did not support growth and was in fact inhibitory.

All the antagonists tested with the exception of aminopterin, methopterin and 4-aminoteropterin inhibited *S. faecalis*/A at various concentrations of or below 500 γ/ml. (Table I). It was also noted (Table I) that the 4-amino group, the 10-methyl group, or the presence or absence of a substitute for the glutamic acid moiety slightly affected the response of *S. faecalis*/A. The addition of a methyl group in the 10 position of pteroic acid made that compound ineffective as a growth factor above 500 mγ/ml, while pteroic acid was able to support maximum growth at more than 1000 times this concentration.

The PGA analogs that had a 9-methyl radical did not support the growth of *S. faecalis*/A. It was of interest then to test the effects of some of these compounds on *S. faecalis/A* in the presence of PGA, CF, and aminopterin. The data presented in Table II show the inhibition ratios of a series of these and other PGA antagonists with PGA and CF for both *S. faecalis* and *S. faecalis*/A. With *S. faecalis* it was evident that CF exerted a more competitive action on these antagonists than did PGA. With *S. faecalis*/A the reversal was not competitive except in the case of PGA with "9-methyl-PGA" and 9, 10-dimethyl-PGA, and CF with amethopterin and 4-amino-9, 10-dimethyl-PGA; once a limiting concentration of antagonist was reached the addition of more growth factor did not reverse the toxicity.

In a separate experiment with 10-methylpteroic acid it was found that the toxicity for *S. faecalis/A* was reversed by PGA; the inhibition ratio for PGA at 1 mγ was 500 and for PGA at 5 mγ was 800. This represents a 100-fold increase in resistance by comparison with *S. faecalis*.

The observation that aminopterin was almost as effective as PGA in supporting the growth of *S. faecalis/A* led to an experiment in which a comparison was made between these two compounds in regard to their relative effectiveness in reversing the inhibitory action of aninopterin and adenopterin. In the presence of 10 mγ of either PGA or aminopterin it was found that half maximum growth was obtained with 50 γ of aninopterin and 20 γ of adenopterin, with inhibition ratios of 5000 and 2000 respectively.

Comparative studies of morphological, cultural, and physiological characteristics show no change in the resistant culture other than its altered PGA metabolism.

Discussion. *S. faecalis*/A differed from *S. faecalis* (ATCC 8043) in respect to the quantitative utilization of PGA and some PGA analogs, as well as having developed a pathway through which several PGA antagonists could be used for growth. The same CF requirements for both cultures indicated that the alteration in the resistant strain was in the enzyme system which formed CF from PGA(11,12). The PGA analogs that did not support the growth of *S. faecalis*/A were those that had a methyl group in the 9 position. There was a definite increase in resistance to 9-methyl compounds, however, when the amethopterin resistant culture was compared with the parent strain. This appeared to be similar to the situation that exists in mouse leukemia(13,14), and possibly in human leukemia. In mouse leukemia it has been shown that after resistance develops to amethopterin there is a cross resistance to 4-amino-9-methyl PGA, and 4-amino 9-10-dimethyl PGA(13,14).

The exact pathway through which aminopterin, amethopterin, amino-an-fol, and other PGA antagonists pass in the process of becoming growth factors for *S. faecalis*/A is not known. It appears likely, however, that the compounds are in some way converted to PGA which in turn is utilized as a substrate for the formation of CF. The marked decrease in the amount of pteroic acid required for HMG further indicates the increased efficiency of this culture, and suggests that the activity of a constitutive enzyme(s) has also been increased in the development of amethopterin resistance.

Present evidence suggests that PGA antagonists such as methopterin and aminopterin function as inhibitors by blocking the conversion of PGA to CF(5,11), and that aminopterin interferes with the action of CF in metabolic reactions(15). It appears that *S. faecalis*/A has the ability to remove the 4-amino group without difficulty as shown by the fact that aminopterin is an especially effective metabolite. The 10-methyl group is only slightly less easily removed in methopterin and amethopterin but the removal of a 9-methyl group in 9-methyl-PGA or 4-amino-9-methyl-PGA apparently cannot be accomplished by this organism. This may be due to the greater strength of the carbon-carbon bond as contrasted to the carbon-nitrogen linkage in the other compounds.

The enzyme pattern found in the resistant leukemia may be similar to that of *S. faecalis*/A. It seems likely that the resistant cells in both cases are capable of utilizing certain concentrations of the antagonists or have developed modified enzymes which are not as vulnerable to the antagonists. Toxicity may occur when these enzymes are saturated, and therefore due to conversion of the anti-metabolite to a metabolite a much higher concentration of antagonist would be required to exhibit toxicity.

Summary. 1. *S. faecalis*/A, an organism made resistant to amethopterin, grew well on a series of PGA antagonists except those with a methyl group in the 9 position. 2. This culture was also more efficient than the parent strain of *S. faecalis* in the utilization of PGA analogs that served as growth factors for both organisms, with the notable exception of CF. The amount of CF (leucovorin) required for HMG was approximately the same for each strain. 3. Aminopterin was as effective as PGA in reversing the toxicity of aninopterin and adenopterin in the growth of *S. faecalis*/A.

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Thermal Shock in a New Vibrio Phage.*† (19839)

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The effect of exposure to cold on bacteriophage was studied by d'Herelle(1) who found that young phase resists exposure to –180°C for 10 minutes while older phage does not. Rivers(2) demonstrated that a bacteriophage lytic for colon bacilli was inactivated by repeated freezing (–185°C) and thawing, and that the type of diluent influences the percentage of phase inactivated in this manner.

During the course of studies on heat inactivation of a newly isolated Vibrio phage, a sample of lysate was heated for 0.5 hour at 60°C then plunged into an ice bath (2°C) for 1 hour. There was a pronounced drop in titre after exposure to cold; more than 90% of the particles which survived heating at 60°C for 0.5 hour were inactivated during the period at 2°C. Experiments undertaken to investigate this phenomenon are described below.

Materials and methods. Materials and methods were the same as those described by Smith and Krueger(3). An ice bath main-

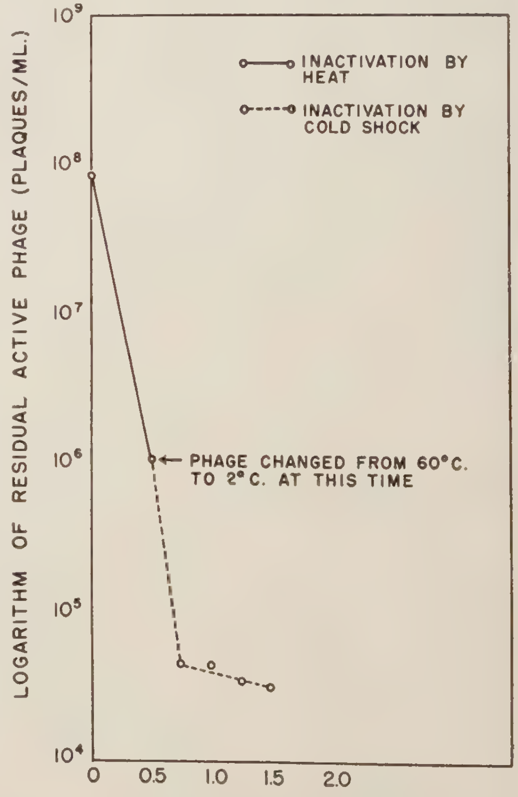

FIG. 1. Course of inactivation due to "Thermal Shock." Samples were titrated after various times at 2°C.

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TABLE I. Inactivation of Phage by "Thermal Shock."

Time and temp. of exposure hr	°C	hr	°C	Titre of residual active phage in plaques/ml
0				8.3×10^7
.5	60			1.2×10^6
		+ .25	2	4.5×10^4
		+ .5	2	4.3×10^4
		+ .75	2	3.3×10^4
		+ 1	2	3.2×10^4

tained at 2°C was used for exposing phage to the effects of cold.

Experimental results. To determine the course of the cold inactivation, phage lysate was heated at 60°C for 0.5 hour. The concentration of active phage remaining at this time was determined; samples of the phage were immediately plunged into the ice bath and at 15-minute intervals one was removed for titration. The greatest drop occurred during the first 15 minutes and comparatively little inactivation took place afterward, (Fig. 1, Table I).

To determine to what temperature phage must be heated before cold shock could be demonstrated, aliquots of phage lysate were held at temperatures from 50°C to 62°C. Samples were taken at 0.5 hour intervals from each of the temperatures used, plunged into the ice bath and kept there for 45 minutes. Samples held at 55°C and at temperatures lower than this were not affected by the sudden chilling. From 56°C to 62°C there was a gradual increase in the inactivation caused by "Thermal Shock", (Fig. 2, Table II).

To ascertain the period of heating required before inactivation by "Thermal Shock" occurs, phage lysate was heated at 60°C and at time intervals samples were plunged into the ice bath where they remained for 45 minutes. Determinations of phage remaining after the various intervals of heating and after subsequent exposure to the cold were made. Using as a criterion the percentage of phage inactivation brought about by "Thermal Shock" it is evident (Table III) that heating at 60°C for 12 minutes is necessary before significant loss due to cooling takes place. Continued exposure to 60°C up to 2 hours does not materially increase the percentage loss due to subsequent cooling.

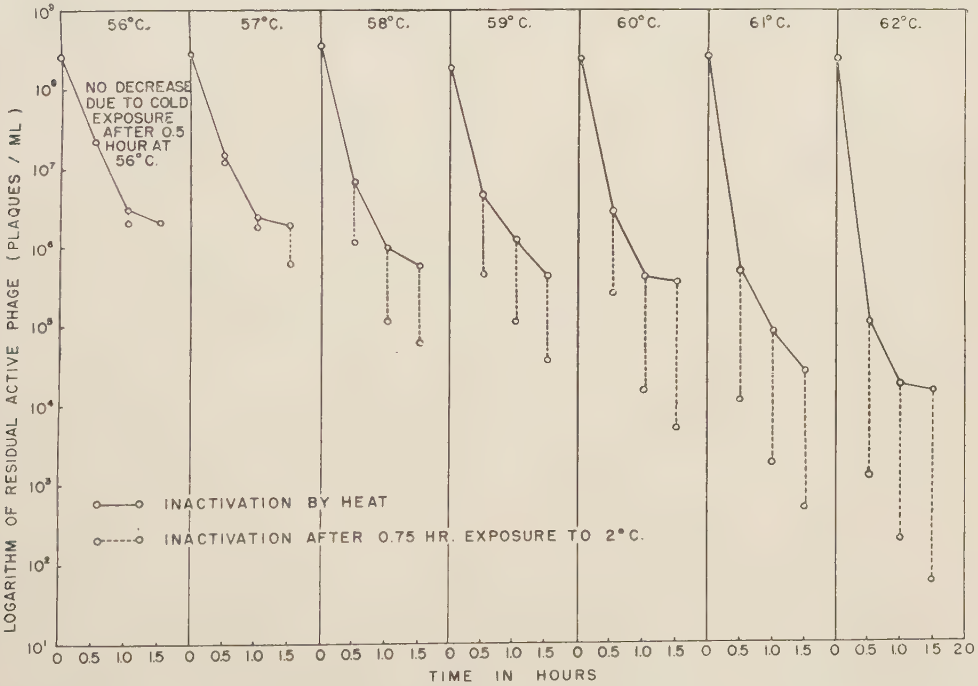

FIG. 2. Effect of time and temperature of exposure on percentage of inactivation by "Thermal Shock."

TABLE II. Inactivation of Vibrio Bacteriophage by Heating at Temperatures from 56°C to 62°C (incl.), and Effect of Temperature of Preheating on Inactivation by "Thermal Shock."

Time of exposure				Titre of residual active phage in plaques/ml—temp. of heat exposure													
Heated hr		hr	°C	56°C	%	57°C	%	58°C	%	59°C	%	60°C	%	61°C	%	62°C	%
0				2.9 x 10^8	—	2.8 x 10^8	—	3.4 x 10^8	—	1.9 x 10^8	—	2.4 x 10^8	—	2.7 x 10^8	—	2.6 x 10^8	—
.5				2.4 x 10^7	—	1.6 x 10^7	—	6.8 x 10^6	—	4.5 x 10^6	—	2.8 x 10^6	—	5 x 10^5	—	1.2 x 10^5	—
.5	+	.75	2	2.5 x 10^7	0	1.53 x 10^7	4	1.3 x 10^6	80	4.5 x 10^5	90	2.6 x 10^5	90	1.2 x 10^4	97	1.4 x 10^3	99
1				3.1 x 10^6	—	2.4 x 10^6	—	9.5 x 10^5	—	1.3 x 10^6	—	4.03 x 10^5	—	8.6 x 10^4	—	1.8 x 10^4	—
1	+	.75	2	2.23 x 10^6	28	1.9 x 10^6	21	1.23 x 10^5	87	1.1 x 10^5	92	1.65 x 10^4	93	1.9 x 10^3	98	2 x 10^2	99
1.5				2.1 x 10^6	—	1.9 x 10^6	—	5.7 x 10^5	—	4.2 x 10^5	—	3.6 x 10^5	—	1.7 x 10^4	—	1.68 x 10^4	—
1.5	+	.75	2	2.1 x 10^6	0	6.1 x 10^5	63	6 x 10^4	89	3.5 x 10^4	92	5.1 x 10^3	97	5 x 10^2	97	6 x 10^1	99+

% = percentage of remaining phage inactivated by cold exposure.

TABLE III. Increase in Inactivation by "Thermal Shock" as the Time Interval of Preheating at 60°C Is Increased.

Min. exp. at 60°C	Min.	°C	Titre residual active phage in plaques/ml	% inactivation
0			3.4 x 10^8	—
3			2.5 x 10^8	—
3	+ 45	2	2.14 x 10^8	14
6			1.2 x 10^8	—
6	+ 45	2	9.4 x 10^7	21
9			2.5 x 10^7	—
9	+ 45	2	2.22 x 10^7	11
12			2 x 10^7	—
12	+ 45	2	2.6 x 10^6	87
15			1.25 x 10^7	—
15	+ 45	2	2.1 x 10^6	83
30			2.8 x 10^6	—
30	+ 45	2	2.6 x 10^5	91
60			4 x 10^5	—
60	+ 45	2	1.7 x 10^4	95
90			3.6 x 10^5	—
90	+45	2	5.1 x 10^3	97
120			1.7 x 10^5	—
120	+45	2	1 x 10^3	99

Investigation of the possibility that this phenomenon might be ascribed to the presence of 3% NaCl was carried out. Phage was resuspended in 1% aqueous yeast extract containing concentrations of sodium chloride ranging from 5% to 0.

Aliquots of each salt concentration were heated at 60°C for 0.5 hour, plunged into the ice bath immediately and held there for 45 minutes. The data tabulated in Table IV indicate that inactivation due to "Thermal Shock" occurs in the presence of sodium chloride in all of the concentrations used, and also in the presence of yeast extract solution to which no electrolyte has been added. "Thermal Shock" also occurred when phage was resuspended in M/15 phosphate buffer pH 7.9 containing 3% sodium chloride and was treated in the manner already described. This was not the case if the suspending fluid consisted of M/15 phosphate buffer at pH 7.9. Samples were plunged into the ice bath after being heated both at 50°C and at 53°C for several different time intervals and no inactivation due to chilling could be observed. Here resistance to heat inactivation was considerably reduced so that the use of the usual time intervals and temperatures was not feasible.

The evidence so far establishes the limits of temperature and time of heating necessary

TABLE IV. Effect of NaCl on Inactivation by "Thermal Shock." Phage was heated at 60° C for 0.5 hr and chilled (2°C) for .75 hr. The menstruum for suspending phage was 1% yeast extract aqueous containing various concentrations of NaCl.

% NaCl	Titre of phage in plaques/ml before treating	Titre residual active phage in plaques/ml after 0.5 hr at 60°	Titre residual active phage in plaques/ml after 0.5 hr at 60°C +0.75 hr at 2°C	% inactivation by "thermal shock"
5	2×10^8	2.69×10^6	1.46×10^5	94
4	1.63×10^8	1.14×10^6	1×10^4	99
3	2.4×10^8	2.8×10^6	2.6×10^5	90
2	1.16×10^8	1.62×10^6	9.9×10^4	94
1	4.26×10^7	6.9×10^5	1.49×10^4	97
.5	4.5×10^7	1.9×10^6	3.2×10^5	83
.25	2×10^7	5.7×10^4	6×10^3	89
.125	1.9×10^7	4.4×10^4	2×10^3	95
0	1.7×10^8	1.6×10^5	6×10^3	96

to prepare the phage particle for inactivation by "Thermal Shock". This phenomenon is not directly dependent upon any one factor in the suspending fluid. However, a suspending fluid which protects the phage from heat inactivation so that it can be held at the necessary temperature for the required time appears to be obligatory.

Further experiments were conducted in an attempt to determine the mechanism involved in the reaction. A search was made for a hypothetical substance which might be formed under the experimental conditions employed to account for phage inactivation. An aliquot of phage lysate was heated at 60°C for 0.5 hour, one portion (A) was plunged into the ice bath immediately, and a second portion (B) was similarly chilled after the addition of unheated phage lysate. If an inactivator were present it was expected that the unheated phage would be inactivated as well as that which had been heated and that the loss of phage per ml would be greater in portion B than in A. As can be seen from Fig. 3a, this did not happen.

Again, an aliquot of phage lysate was heated at 60°C for 0.5 hour, plunged into an ice bath immediately and held there for 15 minutes. Untreated phage lysate was added, and the mixture of previously treated and untreated

	Titre of active phage in plaques/ml
(a) 0 time	1.6×10^8
(b) after 0.5 hr at 60°C	1.8×10^6

A. (b) held at 2°C, 0.5 hr 8.0×10^4 loss 1.7×10^6 plaques/ml	B. (b) $+1 \times 10^6$ phage particles/ml 3×10^6 (b) after 0.5 hr at 2°C 2.09×10^6 loss 1×10^6 plaques/ml

FIG. 3a. Experiment to Detect the Presence of an Inactivator. Phage lysate was heated at 60°C for 0.5 hr. One aliquot was plunged into the cold for 0.5 hr. Untreated phage was added to a second aliquot before it was plunged into the cold. The loss of phage particles was not greater in the sample to which the untreated phage had been added indicating that the cold inactivation is not due to the presence of an inactivator formed during the heating period.

	Titre of active phage in plaques/ml
(a) 0 time	1×10^8
(b) after 0.5 hr at 60°C	1.6×10^6 (loss due to heating 98%)
(c) b. held at 2°C 0.5 hr	1.5×10^5 (loss of remaining phage due to cold exposure 91%)
(d) c. + untreated phage lysate 1.5×10^7 phage particles/ml	1.8×10^7
(e) d. held at 0.5 hr at 60°C	1.86×10^6 (loss due to heating 90%)
(f) e. held at 2°C 0.5 hr	9.9×10^4 (loss of remaining phage due to cold exposure 95%)

FIG. 3b. Experiment to Detect the Presence of an Inactivator. Phage lysate was heated at 60°C for 0.5 hr. Untreated phage was added to the treated lysate. This mixture was heated and cooled in the same manner as the original sample. The percentage loss of phage particles was not greater in the mixture than in the original lysate suggesting that the cold inactivation is due to a change in the physiological state of the phage particle rather than to an inactivator.

TABLE V (a). Experiment to Detect Possible Role of Reactivation in "Thermal Shock."

Time and temp. of exposure							Titre residual active phage	
hr	°C	hr	°C		hr	°C	in plaques/ml	%*
0							1.5×10^8	--
.5	60						1.3×10^6	—
		+ .5	2				3×10^4	97
		+ .5	20	L†	+ .75	2	2.3×10^4	98
		+ 1	20	"			2.2×10^4	98
		+ 1.5	20	"			2.2×10^4	98
		+ 2	20	"			2.2×10^4	98
		+ 2.5	20	"			2.2×10^4	98
		+ 3	20	"			2.3×10^4	98
		+ .5	30	D			3.2×10^4	98
		+ 1	30	"			2.7×10^4	98
		+ 1.5	30	"			2×10^4	98
		+ 2	30	"			2.1×10^4	98
		+ 2.5	30	"			2.2×10^4	98
		+ 3	30	"			2.3×10^4	98

* Percentage remaining phage lost due to chilling.
† L = light; D = dark.

phage lysate was then heated and chilled as was done with the original sample. The course of inactivation of the mixture followed closely that of the original lysate, (Fig. 3b).

These two experiments gave no indication that the hypothetical inactivator is involved in production of "Thermal Shock". Also the fact that "Thermal Shock" occurs in the presence of widely different suspending fluids makes the existence of such a substance seem improbable.

The possibility of aggregation of phage particles in the cold was next considered. Samples of phage lysate were treated by "Thermal Shock". After this, one half were shaken and mixed violently while the other half were mixed gently. Plaque counts of all the samples gave no evidence of significant differences in titre. If aggregates were present they were not broken up by the procedure employed.

It is also conceivable that this phenomenon may represent a situation in which denaturation and reversal of denaturation or photoreactivation are involved. In this case the titres obtained after the samples have been iced would represent the true values for the course of heat inactivation. The difference between the titres obtained from a sample before and after icing would be a measure of the number of phage particles which had become reactivated due to incubation at 30°C or exposure to light before being chilled.

To test this possibility an experiment was performed as follows: Aliquots of lysate were held at 60°C for 0.5 hour. One was chilled immediately and a series of 6 aliquots was kept at 30°C in the dark incubator. One of these was plunged into the ice bath at each half-hour interval during a period of three hours. Another series of 6 aliquots after being heated, was held at room temperature (20°C) and directly exposed to the fluorescent light which was the customary light source in the laboratory. One of these samples was plunged into the ice bath at each half-hour interval over a period of three hours. If reactivation takes place under either of these sets of conditions, no further loss in titre would be expected on chilling, (Table Va & b). However the typical drop occurred; according to this evidence once the phage particle becomes vulnerable to inactivation by "Thermal Shock" it remains so for a period of at least 3 hours.

These results would be expected if the lysate contained two kinds of phage, one more thermostable than the other and at the same time susceptible to inactivation by cold. Attempts to isolate such a phage from among survivors after heat treatment were unsuccessful(3).

Discussion. The phenomenon of inactivation by "Thermal Shock" refers to the decrease in phage titre due to sudden chilling and is in addition to that which occurs during preliminary heating. The conditions of tem-

TABLE V (b). Control Experiment for Table V (a) to Detect Effect of Standing at 20°C and at 30°C on Phage after It Has Been Heated at 60°C for .5 Hr.

Time and temp. of exposure				Titre residual active phage in plaques/ml
Hr	°C	Hr	°C	
0				9.9 x 10^7
.5	60			6.7 x 10^5
		+ 1	20 in L*	6.3 x 10^5
		+ 2		6.1 x 10^5
		+ 3		6 x 10^5
		+ 1	30 in D	6.9 x 10^5
		+ 2		7.4 x 10^5
		+ 3		7.7 x 10^5

* L = light; D = dark.

perature and time required for its occurrence were established. The differences in titres found to obtain between lysates that had been heated and those which had been heated and chilled could be explained only by postulating a loss of active phage when previously heated phage is plunged into the cold bath, *i.e.*, there is no evidence that reactivation of inactivated phage occurs under the conditions of the experiment.

As the time and temperature of the heat treatment is increased the change in titre of the phage when chilled is increased. This is further evidence in favor of a cold inactivation for if reactivation were occurring we would expect the opposite effect. When phage particles are inactivated by heat it may be assumed that the denaturation becomes more permanent as time and temperature of exposure are increased. Reactivation then would become decreased with longer exposure to higher temperatures.

It could be assumed that we are dealing with a mixture of phages, one of which is both thermoresistant and labile to cold. Isolates from phage suspensions previously exposed to heat (and to heat and cold) did not confirm this concept.

If "Thermal Shock" is actually a loss phenomenon in which final exposure to cold somehow causes the inactivation of phage, this step could be ascribed to: a) the development of an inactivator or b) a change in the physiological state of the phage particle brought about by heating and rendering it very susceptible to exposure to low temperatures. Since we have not been able to secure any experimental evidence suggesting the existence of an inactivator the second explanation appears to be the more acceptable of the two. There is no experimental basis for deciding at this time whether thermal shock: a) actually disrupts the phage particles, b) produces changes in the particle such that it cannot be adsorbed to the host cell, or c) alters the phage enough to prevent its participation in the complex reactions requisite for reproduction of phage without destroying its capacity for union with the bacterium.

Summary. 1. The phenomenon of "Thermal Shock" is described. Phage, which was partially inactivated by heating at 60°C for 0.5 hour, showed further inactivation when plunged into an ice bath (2°C). 2. Temperature and time conditions of heating to prepare the phage for inactivation by "Thermal Shock" have been defined. 3. Evidence is presented that "Thermal Shock" is a result of change in the physiological state of the phage particle brought about by pre-heating and does not involve a masked reactivation.

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Encephalomyelitis in the Rat Following Intracutaneous Injection of Central Nervous System Tissue with Adjuvant. (19840)

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By often repeated injections of CNS tissue over a period of several months, Rivers and his associates produced in the rhesus monkey sterile disseminated meningo-encephalomyelitis(1,2). With the aid of adjuvants (paraffin oil containing killed mycobacteria or killed Nocardia asteroides) the rapid production of this experimental malady became possible. Either single or a few injections of CNS tissue with this adjuvant induced encephalitis in the monkey, guinea pig, rabbit, mouse, and dog (3-9).

It is of interest to ascertain whether or not the albino rat is susceptible to the production of this experimental disease since the rat has a low capacity for antibody formation and active anaphylactic sensitization. Neither the phenomenon of Arthus nor skin sensitization to tuberculin has been produced in the rat. However, specific systemic reaction to tuberculin has been demonstrated in this species (10). Since the mouse with similar low capacity for immune response proved to be susceptible to allergic encephalitis, it seemed likely that the rat would not be refractory, although Lumsden(11) failed to induce the phenomenon in rats by subcutaneous injection of homologous brain with adjuvant. In the experiment to be described, homologous spinal cord with adjuvant was injected into the skin.

Procedure. Five male rats (Sherman strain) weighing from 145 to 195 g were used. They were fed Rockland Mouse Diet. The material to be injected was prepared in the following way: Rat spinal cord freed from the meninges was suspended in distilled water containing .25% phenol using a TenBroeck grinder. The suspension contained 33% tissue (wet weight). It was emulsified with an equal volume of a mixture of Arlacel A (1.5 parts) and Bayol F (paraffin oil) (8.5 parts) containing 4 mg (dry weight) of heat-killed and dried tubercle bacilli (Jamaica 22) per milliliter of the mixture. Emulsification was done with a syringe. The rats received 7 simultaneous intracutaneous injections of 0.1 ml of the emulsion, *i.e.,* 16.5 mg of spinal cord, 0.0335 ml phenolized water, 0.0075 ml of Arlacel A, 0.0425 ml of Bayol F and 0.2 mg tubercle bacilli per injection. Three injections were given on the left and 3 on the right side of the back, and one on the anterior surface of the neck. A group of 8 rats served as control. Each rat was given 2 kinds of emulsion: A) water-in-oil emulsion containing tubercle bacilli; B) water-in-oil emulsion containing spinal cord. Seven intracutaneous injections were made, 0.1 ml per site of preparation A on the left and seven 0.1 ml per site of preparation B on the right side of the rats. The amount of spinal cord and tubercle bacilli were the same as in the previous group.

Results. All of the 5 rats of the first group developed flaccid paralysis of the hind legs 11 to 15 days after the injection. Some of the rats had urinary incontinence. The rats were killed when *in extremis.* In the brain and spinal cord there was perivascular cuffing about capillaries and venules; occasional glial nodules were seen. The cells about the vessels were mononuclear cells. No polymorphonuclear leucocytes were found. In the meninges there was slight focal thickening with the acmumulation of mononuclear cells; no polymorphonuclear leucocytes were found. The site of the injection was similar to that found in guinea pigs receiving CNS tissue in water-in-oil emulsion containing tubercle bacilli. There were vacuoles of oil underneath the epidermis surrounded by epithelioid cells, fibroblasts, Langhans giant cells, and a few lymphocytes. There was intense phagocytosis of tubercle bacilli and paraffin oil. In some areas there was necrobiosis of the inflammatory cells attracting polymorphonuclear leucocytes.

None of the control rats developed paralysis or other possible signs of encephalitis. Histological sections of the CNS revealed no pathological changes.

In a subsequent experiment meningo-encephalomyelitis was produced in rats substituting spinal cord of guinea pigs for homologous spinal cord.

Summary. The introduction of homologous spinal cord in a water-in-paraffin oil emulsion containing killed tubercle bacilli into the skin of the albino rat produces mononuclear meningo-encephalomyelitis. The symptoms and pathological changes in the CNS and at the site of injection are similar to those found in the guinea pig.

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Recovery of Type I (Brunhilde) Poliomyelitis Virus from Mouse Brain and Spinal Cord.* (19841)

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Dick reported that type 1 (Brunhilde) poliomyelitis virus in the form of monkey central nervous system tissue inoculated into a variety of rodents could be recovered from their brains at least for 10 days from the time of cerebral inoculation(1). No adaptation of this virus to rodents occurred despite his employment of the method of alternating passage between rhesus monkeys and rodents and passage from rodent to rodent. There was neither clinical nor histological evidence of disease in the rodent hosts studied. It was stated "this is highly suggestive that no multiplication had occurred in the brains of these animals."

This interesting phenomenon was explored as part of a study in this laboratory on poliomyelitis virus-host adaptation. The purposes of this report are to confirm Dick's findings in murine brain and also a) to indicate recovery of virus at a site *distant* to the site of inoculation namely the spinal cord, b) to identify the virus recovered as type 1 virus which had not undergone detectable immunological alteration, and c) to indicate that the biological state of the virus was not such as to interfere with the propagation of a mouse adapted type 2 (Lansing) poliomyelitis virus in such mice.

Materials and methods. Type 1 (Brunhilde) poliomyelitis virus was utilized in the form of a 20% suspension of spinal cord enlargements of monkeys sacrificed at the height of paralysis following thalamic inoculation. This virus pool yielded a titer of $10^{-5.4}$ in monkeys. The mouse adapted type 2 (Lansing) poliomyelitis virus was an aliquot of a pool of infected mouse brains. The number of previous mouse passages approximates 250; average titers of $10^{-3.6}$ are observed with intracerebral inoculation of 0.03 ml in young adult mice. Swiss mice of Harlan stock were utilized in all the observations herein reported. Roller-tube tissue-cultures were utilized for the isolation and identification of poliomyelitis virus. Monkey testicle(2) was used as tissue

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* Aided by a grant from the National Foundation for Infantile Paralysis.

source for fibroblasts and the cytopathogenic effect described by Robbins *et al.*(3) utilized as an indicator for the presence of poliomyelitis virus. The type 1 (Brunhilde) antiserum used was obtained from monkeys hyperimmunized with the prototype Brunhilde virus. Such serum has not thus far revealed a common antigen among the 3 immunological types of poliomyelitis virus(4). These tests for presence and neutralization of virus were qualitative. Quantitative studies are in progress and will be reported.

Experimental. A. *Inoculation of mice with unadapted type 1 (Brunhilde) poliomyelitis virus.* Twelve mice were inoculated intracerebrally with 0.03 ml of a 20% suspension of the type 1 virus. Five days later the brains of six mice were removed and frozen; the spinal cords of these animals were then removed and frozen. Ten days following inoculation the remaining animals were sacrificed and their brains and spinal cords also frozen separately. The following day the mouse tissues were thawed and 5% saline suspensions of brains and 20% suspensions of spinal cords prepared and inoculated into tissue-cultures.

B. *Recovery and identification of virus in tissue culture.* The suspensions of brains and of spinal cords of the 5-day and 10-day groups of mice were kept separate. Culture tubes containing actively growing fibroblasts of monkey testicular fragments were inoculated either with 0.4 ml or 0.1 ml of the respective suspensions. No appreciable difference was noted with the size of inoculum. Uninoculated culture tubes were kept as tissue controls and the same type 1 (Brunhilde) poliomyelitis virus in the form of monkey spinal cord tissue used in mice inoculated separately into other roller-tubes. Nine days following inoculation, beginning cytopathogenic effect was noted in the tubes under test as well as in the tubes inoculated with 20% suspension of type 1 (Brunhilde) virus. By 13 days following inoculation the cytopathogenic effect was distinct in all tubes under test and was most pronounced in the tubes inoculated with the suspensions of mouse spinal cords. Individual culture fluids were collected separately. In each of the 4 test situations (brains and spinal cords separately at the 2 stated intervals), it was observed that the virus recovered from the mice had not undergone detectable immunological alteration as indicated by the fact that the cytopathogenic effect produced was specifically inhibited by the type 1 antiserum. In other tissue-culture tests such serum inhibited the cytopathogenic effects of homotypic type 1 (Mahoney) virus.

C. *Failure to demonstrate interference in mice harboring type 1 (Brunhilde) virus in their central nervous system.* Two groups of mice (40 in each) were inoculated intracerebrally with 0.03 ml of the type 1 (Brunhilde) poliomyelitis virus as before. Two similar groups were so inoculated with 20% suspension of normal monkey spinal cord. Five days and 10 days later respective test and control groups were inoculated intracerebrally with 16 LD_{50} of the mouse adapted type 2 (Lansing) poliomyelitis virus. No significant differences as regards time of onset or incidence of paralysis were observed.

Discussion. That type 1 (Brunhilde) poliomyelitis virus which is unadapted for murine brain can be recovered from the site inoculated and from a *distant* site in the mouse spinal cord suggests that the phenomenon described by Dick involves circumstances other than passive persistence of a depot of virus. The sequential fate in mice of intracerebral inocula of living agents whether adapted or not is not entirely clear. Schlesinger(5) has observed that only 3 to 10% of western equine encephalomyelitis virus can be recovered from mouse brain one hour after intracerebral inoculation. This is not due to physical leakage around the inoculation site. Cairns(6) observed a similar situation in studying the fate of intracerebrally inoculated bacteriophage in mice even with inocula as small as 0.005 ml delivered by a micrometer-screw syringe. The recovery from spinal cords of the type 1 poliomyelitis virus inoculated into mouse brains indicated that the virus did not merely persist in a dormant fashion at the inoculation site.

The terms "adaptation" and "host-range" are relative. Formerly it was considered that only type 2 poliomyelitis virus strains could be successfully propagated in mouse brains. Recently type 3 poliomyelitis virus(7) has been shown capable of propagation in mice. Quantitative studies may indicate whether modified growth cycles of "unadapted" type 1 poliomyelitis virus do in fact occur in the mouse brain. Infectivity for neither monkey nor tissue-culture fibroblasts is lost in the process.

There was failure to demonstrate interference in brains of mice between type 1 virus and mouse-adapted type 2 virus in the dosages and time intervals herein reported. When *incomplete* growth cycles of "unadapted" influenza virus occur in mouse brain(8) a variety of effects including interference with wholly unrelated viruses(9) may be demonstrated. Failure to demonstrate interference in our experiments is in harmony with previous findings of one of us(10) in which it was shown under the conditions therein employed that interference could not be demonstrated between type 2 (Lansing) virus and fecal and spinal cord materials subsequently(4) shown to be immunologically distinct type 1 viruses. It was also found that two immunologically *related* type 2 strains, *i.e.*, M.V. which is "unadapted" for mice and Lansing virus failed to exhibit interference.

Conclusions. Unadapted type 1 (Brunhilde) poliomyelitis virus inoculated intracerebrally in mice can be recovered 5 days and 10 days subsequently from the spinal cord of such mice as well as from their brains. Such virus does not appear to have undergone immunologic alteration and is neutralized by a type 1 (Brunhilde) monkey antiserum. The biological state of this unadapted virus in the central nervous system of the mice is such that it does not interfere with the propagation of mouse adapted type 2 (Lansing) poliomyelitis in such mice. It is suggested that the phenomenon may represent circumstances other than mere passive persistence of virus in the recipient mouse brain.

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A Turbidimetric Method for Assay of Vi Antigen. (19842)

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Recent studies(1,6) on the purification of Vi antigen from *Escherichia coli* required a rapid quantitative method for determining the antigen content of extracts and partially purified fractions. Since serological assays were tedious and quantitatively uncertain, a turbidimetric method was devised which took advantage of the propensity of acidic mucopolysaccharides and similar lyophils to form fine flocculent suspensions with albumin at low pH. This is analogous to the turbidimetric assay of hyaluronic acid reported by Kass and Seastone(2) and others(3,4). Although the exact chemical nature of this antigen has not yet been ascertained, its acidic nature, high molecular weight, and good correlation between the specific preciptin titers and the optical densities suggested the use of turbidity measurements as a quantitative measure of the antigen.

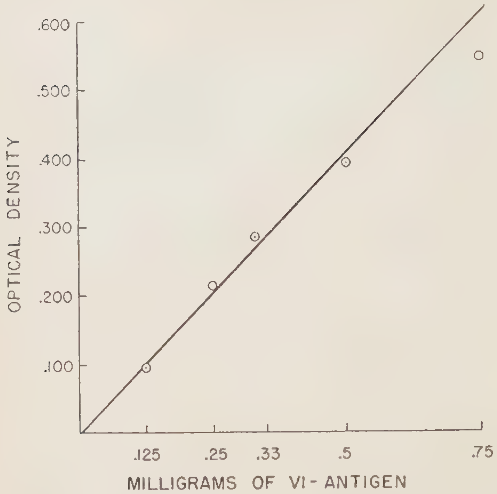

FIG. 1. Optical density of albumin-antigen suspensions for different concentrations of purified Vi antigen, at pH 4 and 26°C. Optical density was measured 10 min. after mixing.

Materials and methods. Acetone dried cells of V form *E. coli,* 5396/38; *Paracolobactrum ballerup,* 7851/39; *Salmonella typhosa,* Ty 2; and *S. typhosa,* 0-901 were prepared by methods detailed elsewhere(5). The last named, lacking Vi antigen, served as a negative control. The principal test organism, *E. coli,* was extracted, fractionally precipitated and concentrated as previously described(1). The purest of these preparations, electrodialyzed Vi antigen (Stage 6), was selected as a primary standard for relating albumin turbidity to immunological activity. Albumin solutions were prepared by dissolving crystalline bovine serum albumin (Armour), 2 mg/ml, in 0.1 N sodium acetate adjusted to appropriate pH values. Ten ml of albumin solution were rapidly mixed with 1 ml of antigen solution, held in a water bath at 26°C for exactly 9 minutes, and then mixed by inversion. Exactly 1 minute later, the optical density of the turbid suspension was measured at a wave length of 6000 Å, in a spectrophotometer previously adjusted to 100% transmission with the buffered albumin. A second sample of the antigen solution was mixed with the buffer solution without albumin, and its optical density was subtracted from the optical density of the antigen-albumin suspension. Antigen concentrations were read from standard curves, Fig. 1, relating mg of antigen to optical density, prepared by treating measured amounts of the selected antigen sample with albumin at pH 4.0. A satisfactory linear relation between optical density and mg of purified antigen was found up to concentrations of 0.5 mg of antigen. Above this value, linearity failed and samples containing more antigen were appropriately diluted.

Of the several conditions necessary for constant reproducible turbidities, acidity was the most critical. Albumin solutions, 2 mg/ml, were mixed with 0.33 mg of purified Vi antigen. After 10 minutes, maximum optical densities were found between pH 4.2 and 4.7, Fig. 2. However, these particles aggregated rapidly to a heavy coagulum with correspondingly rapid changes in optical density. Since this was unfavorable for accurate measurement, pH 4.0 was chosen where the optical density of the fine suspension changed slowly with time. At pH 4.0 and 26°C, 0.33 mg of purified Vi antigen treated with albumin reached a maximum turbidity in about 20 minutes, (Fig. 3). After 6 minutes, however, the optical densities changed more slowly; and a more convenient time of 10 minutes was used without entailing significant errors. Optical densities increased to maximum values more rapidly at high temperatures; while the slower aggregation of micelles at lower temperatures minimized the errors. A satisfactory

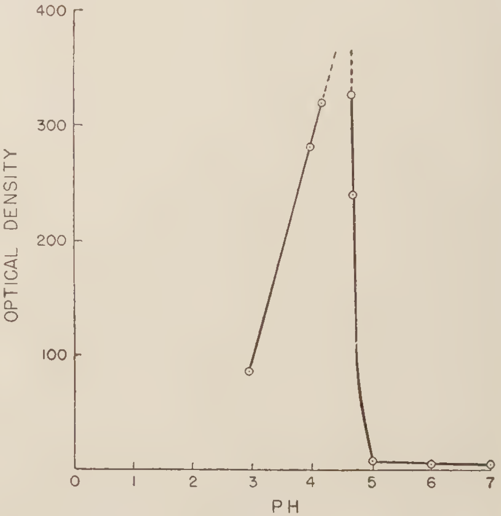

FIG. 2. Optical density of albumin-antigen suspensions, .33 mg purified Vi antigen, at different pH values.

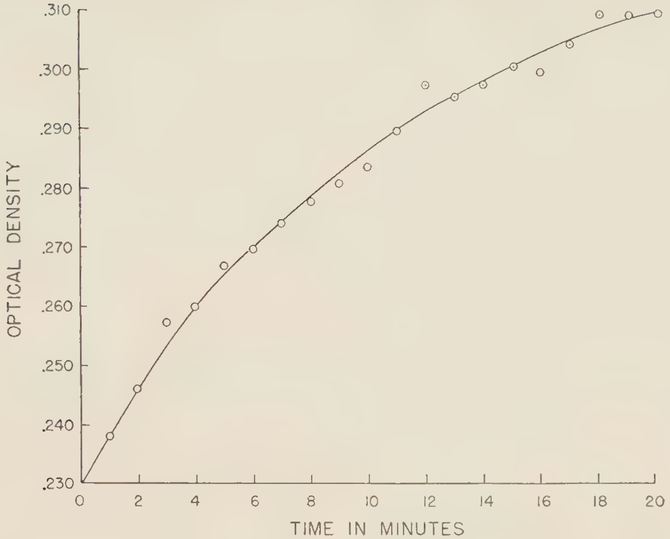

FIG. 3. Change of optical density with time in albumin-antigen suspensions at pH 4 and 26°C. Concentration of purified Vi antigen, .33 mg/ml.

compromise was taken at 26°C. The ionic strength did not have a profound effect under these conditions and distilled water was usually used to dissolve and dilute antigen samples. Two mg of albumin per ml exceeded the amount necessary to produce maximum turbidity with any of the antigen concentrations employed. However, different albumin preparations were found to yield different turbidities with the same antigen solution. Therefore, each new albumin solution was carefully standardized against a standard antigen sample if absolute values were desired.

Many other high molecular weight acids and polymers such as nucleic acids, hyaluronic acid, pectin, hemicelluloses, etc. are known to flocculate with albumin at low pH. Nucleic acids caused an insignificant error with the extracts from *E. coli* and *P. ballerup,* which contained relatively large amounts of Vi antigen. On the other hand, the extracts from *S. typhosa* contained such small amounts of Vi antigen that the contaminating nucleic acid contributed excessive errors and had to be removed by heating the extracts with *N* acetic acid for ½ hour in a boiling water bath and centrifuging.

TABLE I. Serological Activity and Optical Density of Vi Antigen-Albumin Suspensions.

Purified antigen	Optical density	Precipitin titer
E. coli	.004	1:8
	.026	1:128
	.138	1:256
	.320	1:512
*P. ballerup**	.268	1:256
	.430	1:256
S. typhosa, Ty 2	.073	1:64
	.380	1:512
0-901	.013	0

* Precipitin tests with antisera from rabbits immunized with *E. coli.*

Results. The procedure has given a satisfactory turbidimetric measure of Vi antigen in solutions and extracts. The relation of optical densities to specific precipitin titers is shown in Table I. The precipitin tests were carried out in the usual manner with antisera from rabbits immunized with *P. ballerup.* Within the limitations of this test, a quantitative relationship is evident. The turbidity method, however, has the great advantage of yielding a continuous series of quantitative values. It is conceivable that this procedure might also be of value in screening other bacterial extracts for the presence of similar acidic polymers and in facilitating their purification and identification.

Summary. It has been found that the Vi antigen content of extracts and partially purified fractions of *Escherichia coli, Paracolo-*

bactrum ballerup, and *Salmonella typhosa* can be determined by a turbidimetric procedure herein described.

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Production of Fever and the Shwartzman Phenomenon by Native Dextran.* (19843)

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Dextrans are polysaccharides, macromolecular polymers of glucose produced by bacteria, predominantly those belonging to the genus *Leuconostoc,* in culture media containing sucrose. These substances are presently of interest because of their use after partial hydrolysis as so-called plasma volume expanders.

The present report is concerned with some of the reactions noted after injection of unhydrolyzed or "native" dextrans in rabbits. These include leukopenia, fever, and the Shwartzman reaction.

Materials and methods. White male rabbits of mixed breed weighing 2-3 kg were used. Bacterial substances injected intradermally to prepare animals for the Shwartzman reaction were P-25 polysaccharide from *Serratia marcescens* (obtained from Dr. M. J. Shear) and a filtrate of agar-washings from a culture of *S. marcescens* prepared by the method of Shwartzman(1). The intradermal dose of P-25 was 0.1 mg in 0.5 ml of physiologic saline solution and of filtrate 0.5 ml of a 1:10 dilution. Leukocyte counts were made on blood obtained from fresh cuts over a small vein in the ear. In certain instances, cover-slip smears stained with Wright's stain were examined for differential leukocyte counts. All fever tests were conducted in an air-conditioned room using methods previously described(2). Several native dextrans obtained through the courtesy of Dr. Walter L. Bloom were used. These included samples from lots N-112 and N-316 from Commercial Solvents Corporation, lot 84506A1 from another commercial source, and three lots of crude dextran designated I, II, and III prepared in Dr. Bloom's laboratory at Emory University. In addition, a few experiments using unhydrolyzed dextran LM512, obtained from Dr. Virginia Whiteside-Carlson at the University of Alabama were done. Dextrans were injected in a solution of 25 mg/ml in physiologic saline. Two specimens of clinical dextran were employed for comparative experiments. These were that produced by Commercial Solvents Corporation and "Macrodex", a Swedish product (Pharmacia Laboratories). All glassware and needles were sterilized for 2 hours at 170° C to destroy any contaminating bacterial pyrogen. Saline solutions were tested frequently in rabbits and were always pyrogen-free.

Experimental. Leukopenia. The injection of as little as 10 mg of unhydrolyzed dextran produced transient leukopenia followed by leukocytosis in rabbits. With doses of 100-200 mg, leukopenia due to decrease in circulating heterophiles was invariably present within 5 minutes after injection and persisted for as long as 2 hours, finally giving way to heterophile leukocytosis after a few hours. This alteration of the peripheral leukocytes is

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* This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

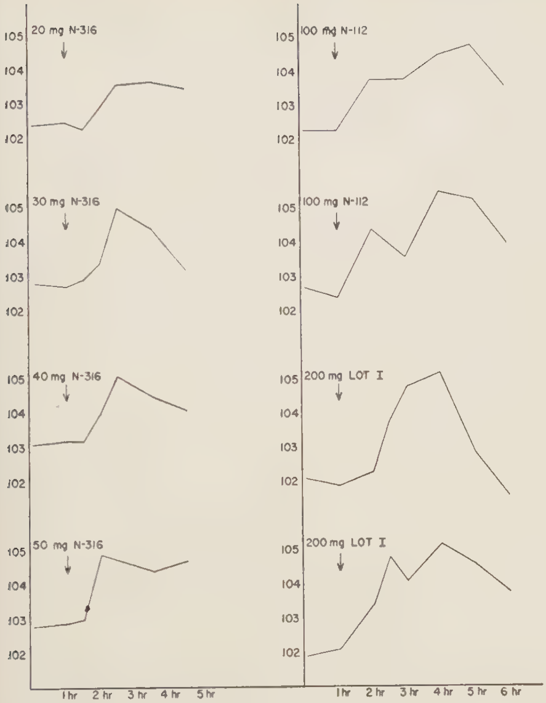

FIG. 1. Temperature responses of individual rabbits after inj. of various amounts of native dextrans. Numbers on left indicate rectal temp in °F.

entirely similar to that which has been noted by many observers after injection of pyrogenic substances derived from Gram negative bacteria(3) or after injection of various polysaccharides such as starch, acacia, and glycogen (4-7). In their studies of transient leukopenia, Essex and Grana(6) mention using a specimen of dextran which they found to be a potent material in producing a fall in circulating leucocytes.

Fever. Although mild temperature elevation occasionally followed the injection of 10 mg of crude dextran, with most of the samples tested, significant febrile responses occurred in the dose range of 20-30 mg. The injection of 100-200 mg of dextran produced a brisk fever beginning within 30 to 60 minutes, rising rapidly to a peak and falling off gradually over a period of several hours. Secondary rises after 3-4 hours were not uncommon. Fig. 1 illustrates typical temperature curves in individual animals after injection of dextran. The febrile response of rabbits to injection of native dextrans corresponds in every respect to that which is seen after injection of pyrogens or endotoxins from Gram negative bacteria(3) raising the possibility that the observed thermogenic effect is a result of contamination by ordinary pyrogenic substances. Although the fact that the fever-producing dose of all the seven dextrans tested was 10-30 mg is a strong argument against random pyrogen contamination in view of the variety of sources of these samples, other experiments designed to exclude this possibility were performed. Briefly, it was found that hydrolysis of native dextran by boiling at an acid pH followed by reprecipitation with alcohol resulted in loss of the temperature-elevating effect. Dextrans deliberately contaminated by bacterial pyrogen (typhoid vaccine or Pyromen, a *Pseudomonas* concentrate) retained pyrogenic activity after similar treatment.

Shwartzman reaction. The intravenous injection of 50 mg of unhydrolyzed dextran 24 hours after intradermal preparation with P-25 or *S. marcescens* filtrate resulted in the appearance of hemorrhage at the prepared sites in about 50% of rabbits tested. Using 200 mg intravenously, large areas of hemorrhagic necrosis were produced in more than 95% of the animals. In general, the areas of hemorrhage were much larger than those we have been able to produce using bacterial materials or glycogen in this laboratory. Dextran was not effective as a skin-preparatory agent. The intravenous injection of dextran or bacterial filtrate produced no hemorrhage in animals given dextran intradermally in amounts up to 50 mg.

Two features of the Shwartzman reaction elicited by intravenous injection of dextran deserve comment. As has been noted with other polysaccharides such as starch and glycogen(8,9), hemorrhage at the prepared skin site often appeared within 30 minutes in contrast to the delay of 2-4 hours when bacterial filtrates are used. Furthermore, animals exhibiting multiple large hemorrhagic reactions elicited by injection of dextran were remarkably free of evidences of systemic intoxication, such as prostration, conjunctival injection and diarrhea, so common after intravenous injection of bacterial toxins. A similar absence of systemic reaction was noted by Freund(8) in animals given starch to elicit the Shwartzman reaction.

Effect of clinical dextrans. In comparative tests using partially hydrolyzed dextrans prepared for clinical use as plasma substitutes, none of the described effects were observed. The average molecular weight of these clinical preparations is approximately 75,000 while that of native dextrans is probably several million. Whether the observed differences are a function of molecular weight alone, specific linkages or configurations is uncertain and is a subject for future investigations.

Discussion. The results reported here indicate that unhydrolyzed dextrans are capable of producing effects similar to those of toxins from Gram negative bacteria when injected into rabbits. The leukopenia and fever after dextran are entirely similar to those seen after injection of endotoxins, but in its elicitation of the Shwartzman phenomenon and its inability to prepare skin for this reaction, it resembles other polymers of glucose such as glycogen and starch.

Stetson(9) has recently pointed out that antigen-antibody reactions and injection of glycogen, both procedures which will elicit the Shwartzman reaction in prepared skin, result in transient leukopenia similar to that observed after toxin administration. The leukopenic effects of antigen-antibody reactions and of glycogen have been noted by many observers(3,4-7) and, the possibility that this leukopenia, which is due to trapping of leukocytes in the capillary bed, is of significance in the production of skin-hemorrhage is strongly supported by Stetson's experiments.

We have been unable to find any account of systematic testing of materials which will elicit the Shwartzman reaction or leukopenia for their ability to produce fever and are at present investigating this possibility. Thus far, in a limited number of experiments with various preparations of glycogen, it has become evident that not all glycogens will elicit the Shwartzman phenomenon or leukopenia and, in our experience, all samples which have proven active in eliciting the Shwartzman reaction have also been pyrogenic.

There is no evidence that the effects of native dextran in rabbits reported here are related to the untoward reactions which are occasionally seen with the clinical use of hydrolyzed dextrans.

It seems possible that further investigations of the activity of crude dextran may yield information of value in determining the mode of action of bacterial endotoxins, many of which are predominantly polysaccharide in nature.

Summary. It has been found that the injection of crude or native dextrans in rabbits produces leukopenia and fever. Evidence is offered that these effects are not due to contamination by bacterial pyrogen. Dextran is also capable of eliciting the Shwartzman reaction in animals prepared by intradermal injection of bacterial toxin. The reaction elicited by dextran resembles that seen after injection of starch or glycogen in its early appearance and in the lack of systemic manifestations of intoxication in the animals tested.

Mrs. Frances Brown and Mrs. Anne McManus gave technical assistance in this work.

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Constant Treatment Technic for Inducing Antibiotic Resistance in Streptococci.* (19844)

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The biochemical and biological properties of beta hemolytic streptococci resistant to various antibiotics have been under study in this laboratory for some time(1-7). Essential to this work has been the production of resistant organisms with a minimum of time and effort and with assurance of the integrity of the strains. Heretofore, the technic utilized was the serial transfer of organisms on blood agar plates containing progressively increasing concentrations of antibiotic. When several strains were subcultured simultaneously on the same plates, the obvious hazard existed of mixing strains by inadvertent substitution, by confluence of growth or by spattering while inoculating. Resistance was also difficult to maintain if plating sequences were interrupted for periods exceeding 3 days.

In the present report a new technic is described in which the bacterial strain was confined in a semi-permeable porous vessel suspended in sterile liquid medium containing antibiotic. This permitted the continuous action of antibiotic on the growing culture and the free exchange of nutritive and catabolic substances. The method was devised to ensure the integrity of the stain throughout the period of the experiment and to determine whether or not antibiotic resistance could be induced more rapidly and with greater ease than with the laborious plate-to-plate technic. This method is designated the constant-treatment technic.

Materials and methods. An inverted hollow porcelain filter candle[‡] was suspended in a 250-ml centrifuge bottle containing 100 ml of Todd-Hewitt broth. An inoculum of 0.1 ml of a 24-hour broth culture of streptococcus was placed in the center of the candle and streptomycin, aureomycin, bacitracin or penicillin was added to the external medium immediately or 24 hours later. The system was incubated at 37°C. Samples were removed at intervals from within the filter by means of a platinum loop and the sensitivity of the organism determined by means of the whole-plate technic. One strain each of group A, B, and C beta hemolytic streptococci, previously studied by the plate-to-plate technic (1-7) and designated as S3, T7 and U1, respectively, were employed. Streptomycin, aureomycin, bacitracin, and penicillin of single lots and freshly prepared in physiological saline were used both in the experimental systems and in the sensitivity test plates. The systems were usually set up in groups of 3 for each strain and each antibiotic with the initial concentration of antibiotic constant for all; one was kept as a control and 2 received subsequent increments of antibiotic, the exact amount being based upon the sensitivity of the sample of organisms most recently withdrawn. Additions of antibiotic to the external medium were made at intervals of one to 10 days, but no nutritive broth was added to the systems. The range of initial concentrations and the variations in the subsequent additions are given in Table I.

Results. A. *Comparison of the plate-to-plate technic with the constant-treatment technic.* The results obtained by the constant-treatment and the plate-to-plate methods in represenative experiments are given in Table II. With the former, in most instances, resistance was induced to streptomycin, aureomycin, and bacitracin in streptococci to the same or greater magnitude than obtained with the latter. In every instance considerably less time was required for this transformation. In the case of penicillin, however, no significant change in resistance was induced regardless of the concentration of antibiotic employed, the subsequent additions of

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[‡] Selas No. 02 porosity with maximum pore size of 0.85 μ.

TABLE I. Antibiotic Concentration in External Medium.

	Streptomycin, μg/ml	Aureomycin, μg/ml	Bacitracin, u/ml	Penicillin, u/ml
Initial conc. of antibiotic				
Range	5–100	.08–5	.005–5	.005–.1
Optimal range*	5–25	.1–.5	.005–1	—
Subsequent additions				
Range	10–10000	.08–80	.005–80	.005–1
Optimal range*	10–500	1–15	1–30	—

* Optimal range: the range of conc. of antibiotics at which an increase in resistance resulted.

TABLE II. Constant-Treatment vs. Plate-to-Plate Technic for Production of Resistant Streptococci.

Antibiotic	Whole-plate technic MFC*	Days†	Constant-treatment technic MFC	Days
Group A (S3 strain)				
Streptomycin	140	60	480	43
Aureomycin	6	60	10	11
Bacitracin	40	60	3.3	11
Group B (T7 strain)				
Streptomycin	400	60	400	20
Aureomycin	12	60	10	6
Bacitracin	15	60	200	13
Group C (U1 strain)				
Streptomycin	3000	57	300	24
Aureomycin	10	60	10	11
Bacitracin	30	60	60	32

* Max fold change in resistance.

† No. of transfers reported in previous publications(1-7) converted to avg time to accomplish this change.

antibiotic or the length of time the organism was exposed to the drug.

B. *Effect of concentration of streptomycin and duration of exposure on induced resistance.* On occasions, group A, B, and C organisms became highly resistant to streptomycin after exposure to concentrations of streptomycin that were sub-inhibitory for the parent strains. A resistance far greater than the cumulative amount of streptomycin to which the organisms had been exposed was reached consistently for members of each group. The early response of identical inocula to similar constant-treatment conditions was not uniform. In one system, for example, employing the group C organism, an extremely low concentration of streptomycin induced a high degree of resistance (60-fold within 24 hours) while no resistance was evident in an identical member of the pair. The final sensitivities, however, after 45 days of treatment and frequent additions of streptomycin, were the same, *i.e.*, greater than 12,000 μg/ml, or a maximum fold change of 240. In another experiment on the same group C organism, this degree of resistance was reached in 24 days with only the initial sub-inhibitory quantity of streptomycin and no subsequent additions. At the completion of this experiment the concentration of streptomycin in the sterile external medium had dropped to less than 1/16th of the inhibitory level for that organism.

Discussion and conclusions. Employing the constant-treatment technic, strains of group A, B, and C beta hemolytic streptococci became resistant individually to streptomycin, aureomycin, and bacitracin. The magnitude of the acquired resistance compared favorably with that obtained by the plate-to-plate method. The advantages of complete bacterial isolation during study, as well as more rapidly induced resistance, make the constant-treatment technic the method of choice. In contrast to these results, penicillin resistance was not acquired using this method. This may mean that penicillin has a different mode of action or that the conditions of the experiment were not suitable. Repeated efforts with varying conditions suggest that the latter explanation is less likely to be the correct one.

The lack of uniformity in the early response of beta hemolytic streptococci to similar constant-treatment conditions in the streptomycin studies can be explained in terms of genetic selection by the presumption that even in samples of inocula drawn at random from the same culture the distribution of resistant mutants varies(8). The fact that the final resistances, after a long period of treatment, are the same supports this presumption. However, the acquisition of high resistance after brief exposure to sub-inhibitory concentrations and the progressive acquisition of the maximum resistance in an environment where the concentration of streptomycin dwindles from a sub-inhibitory level to still lower values are not as readily explained by genetic selection.

No concise range for the management of the constant-treatment technic systems can be given. However, in a given series, involving the same organism and the same antibiotic, the maximum fold change will be virtually equal, although reached, perhaps, by one system in a shorter period of time and/or with exposure to smaller concentrations of antibiotic.

Summary. 1. A new method is described for the production of resistant bacteria by continuous exposure to an antibiotic in a liquid environment. 2. The constant-treatment technic rapidly produces streptomycin-, aureomycin-, or bacitracin-resistant variants of an isolated streptococcus, but fails to produce any significant decrease in streptococcal sensitivity to penicillin. 3. The responses of streptococci to varying degrees of exposure to streptomycin are discussed.

The authors wish to express their indebtedness to Dr. C. Phillip Miller for many helpful suggestions on this study.

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Effects of Antibiotics in Mice on Weight and Thorium Dioxide Uptake of Spleen and Liver.* (19845)

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It has been demonstrated in a number of studies(1-3) that the antibacterial host defense mechanisms play a supporting role in the antibiotic treatment of bacterial infections. In addition to independent participation of the host defense mechanisms, some studies suggest(4,5) that certain antibiotics may act directly upon the cellular defenses of the body to increase their effectiveness. It is also possible that antibiotics may, through direct action decrease the effectiveness of certain host defense mechanisms. In this regard Munoz and Geister(6) have shown that aureomycin inhibits the phagocytic activity of leukocytes of human blood. Lepin, Barski and Maurin(7) have observed that both aureomycin and chloramphenicol, in concentrations attainable in the body, retard the rate of growth of fibroblasts.

In the present investigation several antibiotics were tested for their effect, whether of enhancement or inhibition, on the phagocytic activity of the reticulo-endothelial cells of the liver and spleen of the mouse. The ability of these cells to take up ThO_2 from the circulating blood was used as an indication of their phagocytic ability.

Materials and methods. Laboratory albino mice of mixed sexes were used in all experiments. Prior to each experiment the mice,

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TABLE I. Effect of Antibiotics on Weight and ThO_2 Uptake of Mouse Spleens.

Treatment and dose per 12 hr	Avg spleen wet wt, mg	P for wt diff.	mg ThO_2/g dry wt	P for uptake diff.	mg ThO_2/spleen
P,* 10000 units	133	$<.7$	120	$<.3$	4.10
S	127	—	124	—	3.86
C, 2 mg	143	$<.2$	113	$<.01$	3.83
St, 2 mg	148	$<.3$	114	$<.8$	4.10
S	159	—	116	—	4.32
A, 2 mg	126	$<.05$	131	$<.05$	4.15

* P = penicillin; S = saline; C = chloramphenicol; St = streptomycin; A = aureomycin.

weighing 24 ± 4 g, were pooled and placed into groups at random. Each group contained from 15 to 20 mice; an equal number of animals was included in each experiment for control. Antibiotic treatment with arbitrarily chosen high concentrations was begun 4 hours before the intravenous injection of 0.1 ml of Thorotrast.[‡] Subsequent injections were given every 12 hours for a total of 7 antibiotic injections. All antibiotics were dissolved or suspended in saline. A small amount of Tween 80 was used to help suspend the insoluble antibiotics and the same amount was added to all other solutions. The antibiotic injections were given subcutaneously in a volume of 0.25 ml. Mice in the control groups received the same volume of saline plus Tween 80 according to the above schedule. Seventy-two hours after the injection of Thorotrast the mice were sacrificed by ether anesthesia, their livers and spleens removed and the wet weights of these organs determined by weighing 3 or 4 organs in tared beakers. After weighing, the organs from a group were pooled, minced with scissors and thoroughly mixed. Aliquots not exceeding 400 mg dry weight from this pool were added to tared beakers, dried, weighed and the ThO_2 content determined according to the technic described by Gordon and Katsh(8). The accuracy of the analytical procedure was checked by running analyses on known amounts of ThO_2. One ml of Thorotrast diluted 1:10 was added to each of a series of beakers containing a small amount of normal rabbit liver. The series of 6 values obtained varied between 22.0 mg and 23.3 mg with a mean of 22.7 ± .19 mg of ThO_2. According to the manufacturers, 0.1 ml of Thorotrast contains 24-26 mg of ThO_2. Errors resulting from variation among lots of Thorotrast were minimized by using one sample throughout a given experiment. Results of ThO_2 analyses were expressed as mg ThO_2 per gram dry tissue. Five independent analyses were made on each tissue pool. The significance of differences in ThO_2 uptake between treated and control groups was determined by the "t" test as described by Fisher(9). The significance of changes in weight resulting from treatment was estimated in the same manner. The antibiotics used in this investigation were: sodium penicillin G; dihydrostreptomycin sulfate; aureomycin hydrochloride[§] and pure chloramphenicol.[‖]

Results. The effects of singly administered antibiotics on the spleen weight and the ThO_2 uptake of splenic tissue are given in Table I. It is seen from Table I that chloramphenicol caused a significant reduction in the uptake of ThO_2 per unit weight of tissue; however the average weight of spleens from chloramphenicol treated mice was greater than that of control mice and this weight change may account for the apparent decrease in ThO_2 uptake. Similarly, the significant reduction in spleen weight produced by aureomycin may account for the increase in ThO_2 uptake observed with this antibiotic. In the spleen analyses all of the material in each organ pool was analysed and hence the amount of ThO_2 taken up by each spleen can be calculated. These values, shown in the right-hand column of Table I do not suggest that either chloramphenicol or aureomycin had any marked effect on the uptake of ThO_2 per spleen.

The effects of antibiotics singly and in

[‡] Thorotrast is a stabilized colloidal solution of ThO_2 (24-26% by volume). This chemical was generously supplied by Heyden Chemical Corp., New York.

[§] Generously supplied by the Lederle Laboratories.

[‖] Generously supplied by Parke Davis and Co.

TABLE II. Effect of Antibiotics on Weight and ThO_2 Uptake of Livers.

Treatment and dose per 12 hr	Avg liver wet wt, g	P for wt diff.	mg ThO_2/g dry wt	P for uptake diff.
St,* 2 mg	2.13	<.4	29.4	<.3
S	2.30	—	28.2	—
A, 2 mg	1.59	<.01	31.7	<.7
S	1.63	—	30.9	—
P, 10000 units	1.67	<.2	34.7	<.01
S	1.79	—	30.3	—
C, 2 mg	1.77	<.8	33.6	<.05
A, 2 mg + C, 2 mg	1.81	<.01	30.9	<.02
+ St, 2 mg	1.74	<.01	35.4	<.001
+ P, 2 mg	1.71	<.01	32.4	<.01
S	2.07	—	24.2	—
C, 2 mg + St, 2 mg	1.95	<.2	25.9	<.5
+ P, 10000 u	1.92	<.2	23.9	<.9
St, 2 mg + P, 10000 u	1.76	<.01	28.1	<.05

* St = streptomycin; S = saline; A = aureomycin; P = penicillin; C = chloramphenicol.

combinations of two upon the weight and ThO_2 uptake of livers are given in Table II. It will be noted that aureomycin alone and all combinations which included aureomycin caused a significant reduction in the wet weight of livers. The combination of streptomycin + penicillin also significantly reduced liver weight although this effect was not noted with either antibiotic alone. In all instances in which antibiotics produced significant changes in the ThO_2 uptake per unit weight of tissue the weight changes produced were in the proper direction to account for such a change in uptake.

Summary. The antibiotics penicillin, streptomycin, aureomycin and chloramphenicol were studied for their effects upon weight and ThO_2 uptake by spleens and livers of mice. These antibiotics were studied singly in the case of spleens and singly and in combinations of two in the case of livers. Aureomycin singly and in combination with other antibiotics caused a significant reduction in the weight of spleens and livers. The combination of streptomycin + penicillin caused a reduction in liver weight. The uptake of ThO_2 was expressed as weight of ThO_2 per unit weight of tissue. Apart from the organ weight changes produced there is no evidence that any of the antibiotics in the concentrations used, had a significant effect upon the ability of either spleens or livers to take up ThO_2.

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Shwartzman Phenomenon. III. Modifications of Nitrogen-Mustard Suppression.* (19846)

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The suppressive action of nitrogen mustard on the Shwartzman phenomenon is well established(1-3) and observations on protection of the femoral marrow(2,3) as well as studies of peripheral blood smears and histologic sections(3), indicate that the mechanism of suppression hinges on the granulocytopenia which regularly follows adequate mustard treatment in the rabbit. Despite the high correlation between the presence of the heterophil granulocyte and the occurrence of the phenomenon, the definitive demonstration of the role of the granulocyte is yet to be presented. In its absence it seems of interest to report on some experiments which further strengthen the correlation noted above. The first of these is based on the observation of Weisberger and Heinle that parenteral cysteine confers marked protection against the leucopenia induced by nitrogen mustard(4). These studies were repeated and extended to examine the effect of cysteine protection against the suppressive action of this substance on the Shwartzman phenomenon.

A second approach to the correlation was found in the accidental observation that the hematological response of the rabbit seemed to be altered by relatively mild inflammation. The latter was induced by sterile bacterial filtrates injected intradermally about the time of the mustard injection. This apparent alteration in response and the attendant effect on the Shwartzman phenomenon were studied by means of daily intradermal injections of colitoxin.

Methods and materials. Male, albino rabbits weighing between 1800 and 3000 g were housed in separate cages and given routine laboratory feed and water *ad lib.* (In a few cysteine experiments, sex was not recorded.) *Nitrogen mustard.* Methyl bis β-chloroethyl amine hydrochloride (HN_2) was injected intravenously. Solutions were freshly prepared in the strength of 1 mg/ml in isotonic saline. Two mg/kg of the agent were injected 4 days before the intravenous provocative injection of colitoxin. *Cysteine protection.* Two g of cysteine hydrochloride were dissolved in about 20 ml of distilled water and neutralized to nitrazine paper with 30% NaOH. The solution was injected intravenously 3 to 13 minutes before the injection of HN_2. In some experiments cysteine hydrochloride was given in doses of 750 mg/kg. *Regular skin preparation used in cysteine protection.* The abdomen of the rabbit was lathered and shaved just prior to the intradermal preparatory injection of 0.5 ml of colitoxin (the sterile supernate of a centrifuged 7-day-old brain-heart infusion broth culture of *E. coli,* containing about 0.4% phenol). *Daily intradermal injections.* The abdominal skin of the rabbit was lathered and shaved shortly after the HN_2 injection. Thereafter the animal received an

TABLE I. Modification of Suppressive Action of HN_2 on the Shwartzman Phenomenon.

Group	Modifying treatment	HN_2	Pos*	Neg	Dead‡
1	Cysteine intrav. before HN_2	2 mg/kg	6	4	2
2	Control sol. intrav. before HN_2	”	0	9†	0
3	Daily intradermal inj. of colitoxin	”	3	7	0
4	” ” ” ” saline	”	0	6	3
5	Controls for groups 1-4	—	10	2	3

* Any unequivocal reaction even petechial.

† Controls sol. consisted of equivalent vol of isotonic saline (5 rabbits); equivalent vol of hypertonic 3.35% saline (2); approx. 1 g/kg of Na ascorbate in 5% sol. (2).

‡ Dead before elapse of 4 hr after provocative inj.

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* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

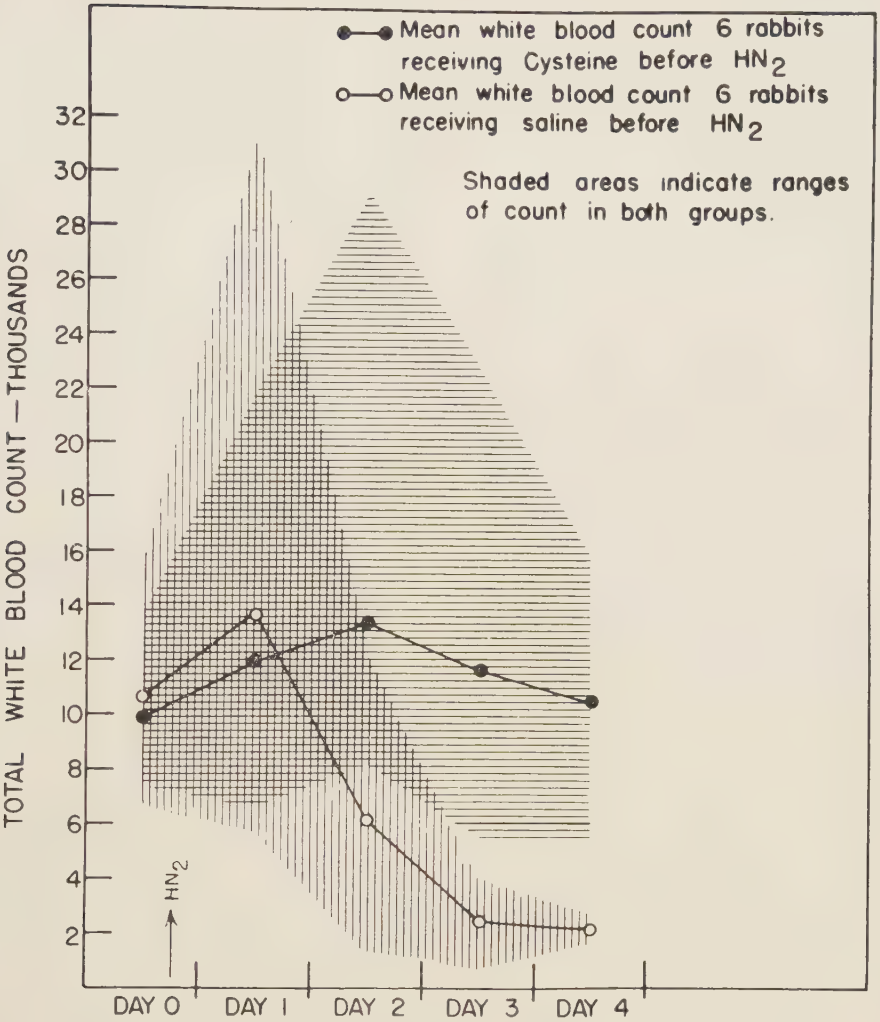

FIG. 1. Protective action of cysteine against HN_2-induced leucopenia.

intradermal injection of 0.5 ml of colitoxin. A similar injection was made in a different area of the abdomen each day, sparing the right upper quadrant for the fourth intradermal injection which served as a routine preparatory injection for the Shwartzman phenomenon, provoked by an intravenous injection of colitoxin on the next day, the fourth after HN_2. Control animals received 0.5 ml sterile isotonic saline in place of colitoxin except for the last, which consisted of a preparatory injection of 0.5 ml colitoxin. *Provocative injections.* One ml/kg of undiluted colitoxin was injected intravenously 4 days after the HN_2 and 24 hours after the preparatory skin injection. In the earlier cysteine experiments agar washings of 24-hour growths of *E. coli* were used in place of colitoxin. *Blood studies.* White blood counts and differential smears were made daily on most HN_2-treated rabbits. Wright's stain was used for the preparation of the differential slides. One hundred cells were counted on each slide.

Results and discussion. The experimental results relating to the Shwartzman phenomenon are summarized in Table I. Fig. 1 indicates that 750-945 mg/kg of cysteine given 3-13 minutes before 2 mg/kg of HN_2, strongly protects against the leucopenic effect of that

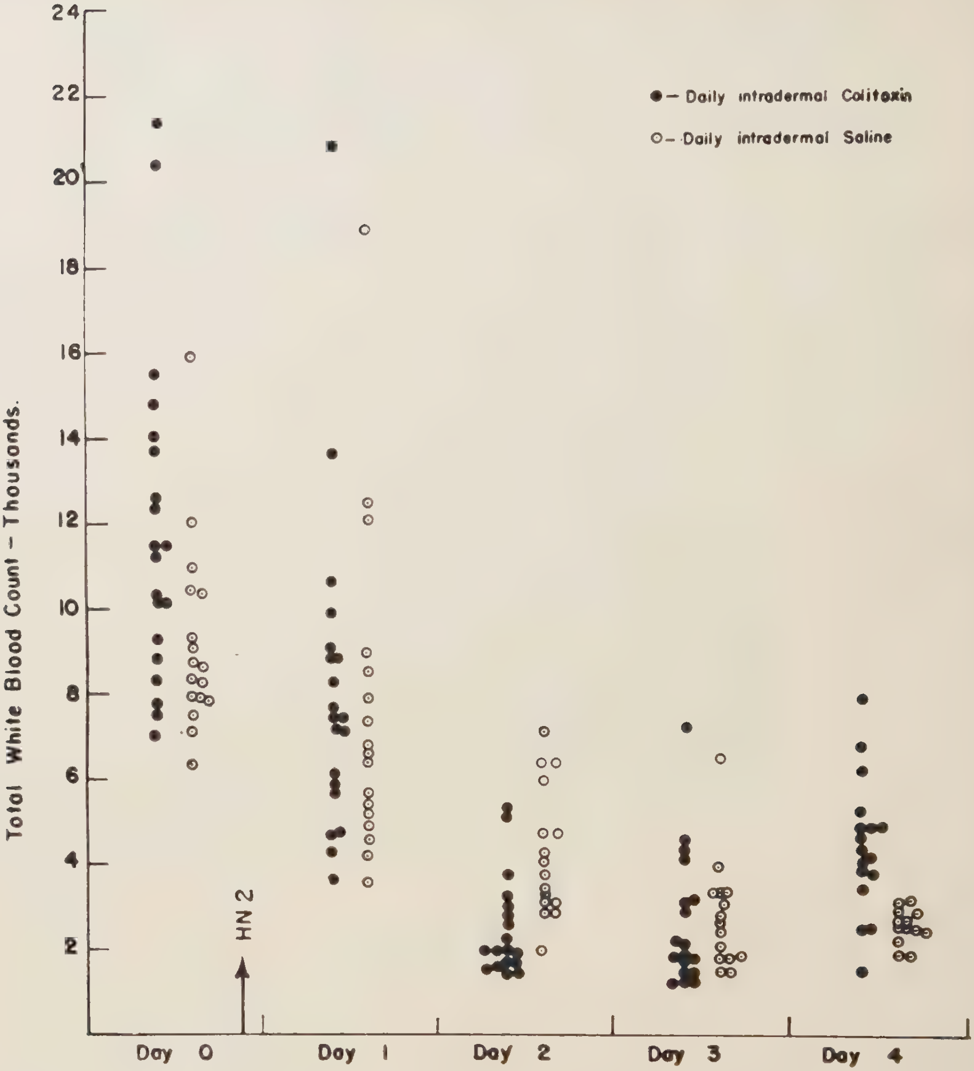

FIG. 2. Modification of mustard-induced leucopenia by inflammation.

agent. Differential smears show that the granulocyte counts remain high, ranging from 1855 to 16800/cmm on the third day, and from 1055 to 8860/cmm on the fourth day. The data in Table I indicate that with the sustained peripheral blood count is seen a concurrent disappearance of the usual suppressive action of the HN_2 on the Shwartzman phenomenon.

Fig. 2 indicates that the daily intradermal injection of 0.5 ml of colitoxin, started after the administration of HN_2, conspicuously alters the hematological response which normally follows 2 mg/kg of HN_2. The regular pattern of leucopenia, following this dose of mustard, is demonstrated in the saline control points in Fig. 2, as has been reported(3,4).

It appears then that the decreased suppressive activity of HN_2 in both the experimental conditions set out above is associated with the presence in the peripheral blood of granulocytes in some quantities at a time when they are ordinarily quite scarce. Further, the slight decrease in the suppressive action of the HN_2 in the second group is associated with a condition in which granulocyte return is also slight.

The last experiment is of interest in another direction, in that a relatively simple process (inflammation induced by intradermal colitoxin) seems to speed appreciably the return towards a normal blood picture in the rabbit receiving HN_2. The significance of the data remains to be determined, but it appears that inflammation may change the rate of development and the rate of discharge from the marrow of the cell or cells that give rise to the normal heterophils in the peripheral blood. This problem is being further studied and details will be presented elsewhere.

Conclusions. 1. The protective action of cysteine against the leucopenia induced by HN_2 is confirmed and extended to the suppressive action of that agent on the Shwartzman phenomenon. 2. The regular pattern of leucopenia may be considerably altered by repeated small inflammatory reactions induced after the administration of HN_2. The suppressive action of the mustard on the Shwartzman phenomenon appears to decrease when such alteration is produced. 3. Additional support for the correlation between the presence of leukocytes (probably granulocytes) and the production of the Shwartzman phenomenon is supplied by these experiments.

Following completion of this manuscript, it was learned that Dr. Ivan Bennett had also completed a study on the protective action of cysteine against nitrogen mustard suppression of the Shwartzman Phenomenon.

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Failure of "Tween 80" to Alter Fecal Fat Excretion in Bile Fistula Dogs. (19847)

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The wetting agent "Tween 80" (polyoxyethylene sorbitan monooleate) has been reported to correct steatorrhea in a child with celiac disease when doses of 40 mg/g dietary fat were given(1). It has also been pointed out that the fat absorptive defect in sprue and in celiac disease resembles that in patients and in experimental animals deprived of bile, in that in these conditions fecal fat excretion is approximately linearly related to dietary fat intake and unabsorbed fat is largely hydrolyzed(2). If the steatorrheas of celiac disease and of biliary deficiency have a common cause, "Tween 80" would be expected to reduce fecal fat excretion in both conditions. Furthermore, if the solubilizing properties of bile salts are chiefly concerned in the facilitation of fat absorption by bile, "Tween 80" would be expected to reduce fecal fat excretion in bile deficient animals since sodium glyco- and taurocholate have been shown capable of so doing(3). The present study was undertaken to test these two implications.

Methods. External bile fistula dogs were fed diets consisting of either "Pard" alone or "Pard" plus 25 g of lard, containing a total of 15 and 40 g of triglyceride fat day, respectively. Either 2 or 4 grams of "Tween 80" were added to each of these diets. The mixtures of food and "Tween 80" were given in one daily feeding for 7 days, the last 5 days of which all feces were collected, blended, and analyzed for petroleum ether soluble material extracted in 5 hours(4). Seven dogs received 2 of the test mixtures and one dog was tested once.

Results. As summarized in Table I, neither dose of "Tween 80" reduced fecal fat excretion below the expected limits for untreated bile fistula dogs.

Discussion. Although "Tween 80" has been shown to reduce fecal fat excretion in 4 partially gastrectomized patients with steatorrhea(6) and to reduce the fat concentration in the feces of 3 out of 4 additional gastrectomy patients(7), it does not appear that

TABLE I. Total Fecal Fat Excretion in Bile Fistula Dogs.

Dietary fat intake, g/day	Total fecal fat excretion				
	Untreated, g/day*	2 g "Tween 80"†		4 g "Tween 80"†	
		No. dogs	g fat/day	No. dogs	g fat/day
15	12.4 ± 1.2 S.E.	2	14.2	5	12.1
40	27.3 ± 3.2 S.E.	3	26.6	5	25.4

* Estimated from previously-demonstrated relationship that fecal fat excretion in bile fistula dogs equals 3.7 g plus .58 ± .08 times dietary fat intake(5).

† Supplied by Smith, Kline, and French.

it will effectively control steatorrhea due to bile deficiency.

The present results fail to support a previous suggestion(2) that the absorptive defect in sprue and in celiac disease may be caused by bile salt deficiency. A dissimilarity in etiology cannot be considered as established, however, because a) the effect of "Tween 80" on fecal fat excretion in the idiopathic steatorrheas has received very limited study; b) the dose of "Tween 80" used in the present study, though up to 3 times that reported to be effective in celiac disease(1), may have been insufficient; and c) the large doses of conjugated bile salts given as desiccated ox bile necessary to reduce effectively the steatorrhea of bile fistula dogs(3) have not been tested on patients with idiopathic steatorrhea.

The present results also fail to support the hypothesis that bile salts play primarily a solubilizing role in their facilitation of fat absorption. Such a possibility has not been disproven, however, in view of the inability to equate the oral dose of "Tween 80" to that of desiccated ox bile, in terms of solubilizing properties, which has been shown to be sufficient to reduce steatorrhea in bile fistula dogs.

Summary and conclusions. Two and 4 g daily doses of "Tween 80" were not shown to alter fecal fat excretion in 8 bile fistula dogs when the mixed diet contained 15 g (5 tests) and 40 g (10 tests) of triglyceride fat per day.

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New Design of Electrophoresis-Convection Apparatus. (19848)

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The electrophoresis-convection cell has been widely used recently in the preparative separation of proteins(1,2,3). This type of cell combines horizontal electrophoresis with vertical convection in a manner that efficiently separates the components of a mixture. The apparatus is much more practical for separations than the Tiselius type of cell, since it can handle larger volumes, requires no fragile glass parts or delicate adjustments, and is more economical.

The apparatus consists essentially of a cell composed of two reservoirs which are connected by a vertical channel with walls of dialysis membrane. The cell is immersed in a buffer solution between two electrodes.

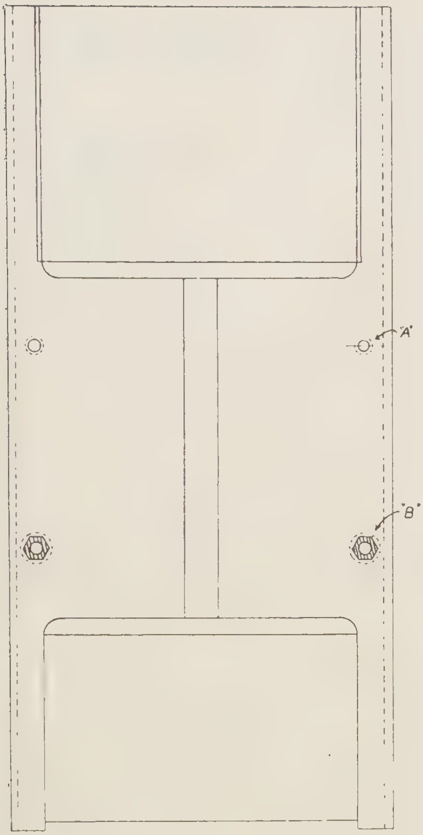

FIG. 1. Face plate. One with bolt-holes as "A." One with nuts as "B."

Earlier designs(4,5) of the apparatus have been of great value in many biological problems. Nevertheless, practical experience has indicated a need for certain improvements: 1) adjustable volume capacity, 2) leak-free construction, 3) aseptic operation. By making use of a new method of construction the apparatus described herein meets these requirements.

The essential feature of the new design is the construction of the cell itself. The entire cell, including channel and reservoirs, is formed of a continuous bag of dialysis tubing which can be sterilized by autoclaving and the contents thereof kept free of subsequent contamination by sterile technics. The bag is supported in the appropriate shape by an external frame. Two electrodes and a container for the buffer solution complete the apparatus. The cell frame is formed of two face plates, constructed of lucite, one of which is shown in Fig. 1. The dimensions of a convenient size for cells of 20 to 250 ml capacity are 12 inches high, 5¾ inches wide and ¾ inch thick. The verticle center slot which exposes the channel is 5 inches by ½ inch. The upper and lower recesses forming chambers for the reservoirs when the two plates are clamped together are 3 inches high, 4½ inches wide and ⅜ inch deep. The two plates are clamped together with 4 stainless steel screws which engage units embedded in one of the plates. To avoid electrolytic action at least one end of the screw must be insulated from the buffer solution. To assemble the cell-and-frame assembly a bag of dialysis tubing of the appropriate size is well soaked in buffer and laid flat upon one of the

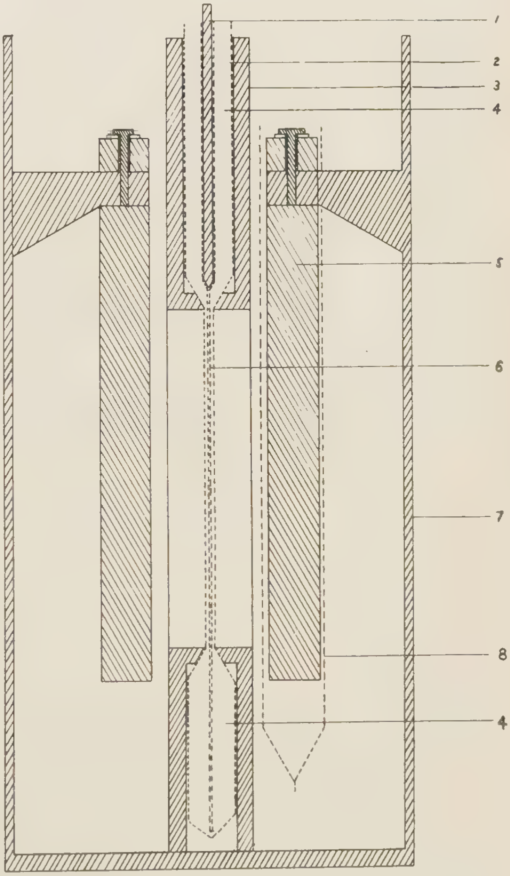

FIG. 2. Electroconvection cell median section. 1. Sliding gate; 2. Solution; 3. Face plate; 4. Reservoir; 5. Electrodes; 6. Channels; 7. Buffer box; 8. Anode diaphragm.

face-plates. The other face-plate is then placed on top and the two are clamped together. When filled with the solution to be treated the bag bulges out into the recesses of the plates, forming the upper and lower reservoirs, and to a lesser extent in the channel slot, forming the channel. It is convenient to use a double length of tubing folded into a U-shaped bag instead of a single bag of tubing tied at the lower end, since this forms two upper reservoirs, two parallel channels, and a U-shaped lower reservoir. The speed of separation is doubled because of the two channels.

The cell frame shown in Fig. 1 may be used with bags of dimensions from 1 inch by 9 inches (measured when the bag is flattened) up to 3 inches by 12 inches, thus giving a range of capacity from 20 ml to approximately 250 ml.

Fig. 2 is a cross-section of the assembled apparatus. The container is a lucite cylinder 6 inches O.D. by 12 inches high provided with inlet and outlet (not shown) for circulation of the buffer. At the upper end of the U-shaped cell is a sliding gate, consisting of a flat plate of lucite with bevelled edges, which can be slid down between the two upper reservoirs into a V-shaped groove to pinch off the tubing at that point. This prevents mixing of the reservoir contents with the channel contents while the former are being removed.

Either graphite or platinum electrodes can be used. With graphite electrodes a cellophane bag must be placed around the anode to prevent contamination of the buffer by carbon particles.

The apparatus is especially useful in fractionating mixtures where maintenance of sterility is important. The procedure consists of autoclaving a length of dialysis tubing closed at each end, assembling the sterilized bag and frame, opening and filling the cell with the sterile solution using sterile precautions, and reclosing the bag during the run. Bacteria cannot pass through the dialysis membrane even though the external buffer is exposed to bacterial contamination.

Summary. An electrophoresis-convection apparatus for fractionating proteins is described which (1) has variable capacity, (2) is of simple leak-free construction, and (3) can be operated under sterile conditions.

The author is indebted to Dr. S. A. Ellison, Dr. T. Shedlofsky, and Dr. H. M. Rose for helpful discussions of this apparatus.

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Improvement of a Hemin Standard by Recrystallization. (19849)

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(Introduced by N. B. Guerrant.)

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The lack of a satisfactory primary standard for the determination of hemoglobin has led to the development of several methods for the preparation of a hemin standard. The present investigation was undertaken to show that the hemin standard proposed by King (5) can be improved by recrystallization. The effect of recrystallization of crude hemin on its purity and stability was studied.

Experimental. Hemin was prepared from both bovine blood and human blood (outdated transfusion blood). Independently prepared samples* of both crude and recrystallized hemin were also used. For the first sample from bovine blood the method of Hans Fischer(3) was used, the increase in purity

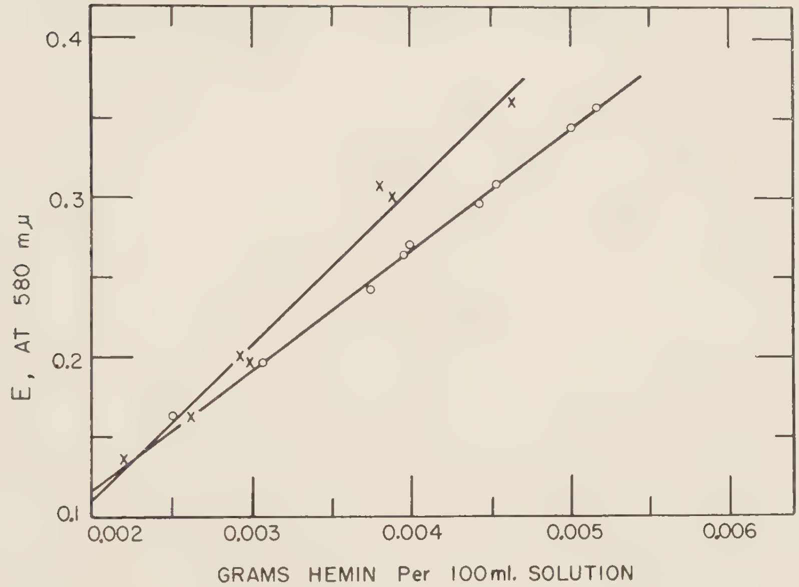

FIG. 1. Extinction vs. concentration. ××—Crude hemin in borate buffer. ○○—Recrystallized hemin in borate buffer.

after each of 4 recrystallizations being studied by determining the percentage of iron in each batch of crystals. A modification of the Delory method(2) was used to prepare hemin from human blood. All hemin samples were analyzed for iron by a micro-modification of the titanous chloride method of Delory(2), and of Wooton and King(6). A modification of the micrometer burette developed by Dean (1), and by Wooton and King(6) was used, the uniformity of the bore being checked by calibration with mercury. Stock solutions of crude and of recrystallized hemin, each containing approximately 3 mg per 10 ml were prepared according to the method of Horecker (4) by dissolving accurately weighed amounts in N/10 borate buffer at pH 9.4. After allowing these solutions to age overnight at about 6°C, they were diluted to give concentrations of hemin ranging from 10 to 50 mg per liter, as recommended by Horecker(4). The conformity to Beer's Law of these solutions was determined by measuring their extinctions at 580 mμ on a DU-Beckman spectrophotometer. The effect of recrystallization of crude hemin on its stability with age was studied by measuring the absorption spectra of these dilute solutions over the range 475-690 mμ at 3 time intervals: immediately after dilution, 16 days after dilution and one month after dilution.

Results and discussion. That recrystallization of hemin causes solutions of it to conform more closely to Beer's Law is shown by the curves in Fig. 1, in which the optical densities at 580 mμ of different concentrations of crude and of recrystallized hemin are shown. Further evidence that the purity of hemin is improved by recrystallization is presented in Table I, the data showing that the iron content is increased by recrystallization.

The variations with respect to wavelength of the extinction coefficients $\left(E_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}\right)$ of 4 different preparations of crude hemin are

TABLE I.
Iron Content of Crude and Recrystallized Hemin.

Sample	% Fe
Crude crystalline bovine hemin (authors)	7.83
1st recrystallization ” ” ”	8.23
2nd	8.33
3rd	8.47
4th	8.45
Crude amorphous bovine hemin (Armour lab.)	6.91
Recrystallized ” ” ”	8.53
Crude crystalline human ” (authors)	8.25
Recrystallized ” ” ”	8.44

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* These samples were contributed by the Armour Laboratories and by the Department of Biochemistry at the University of Buffalo.

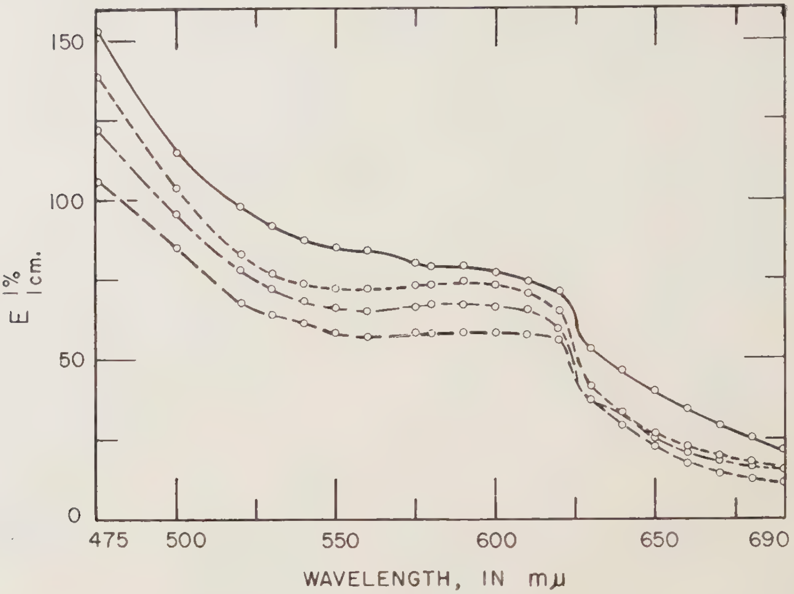

FIG. 2. Comparison of absorption spectra of various crude hemin products in borate buffer.

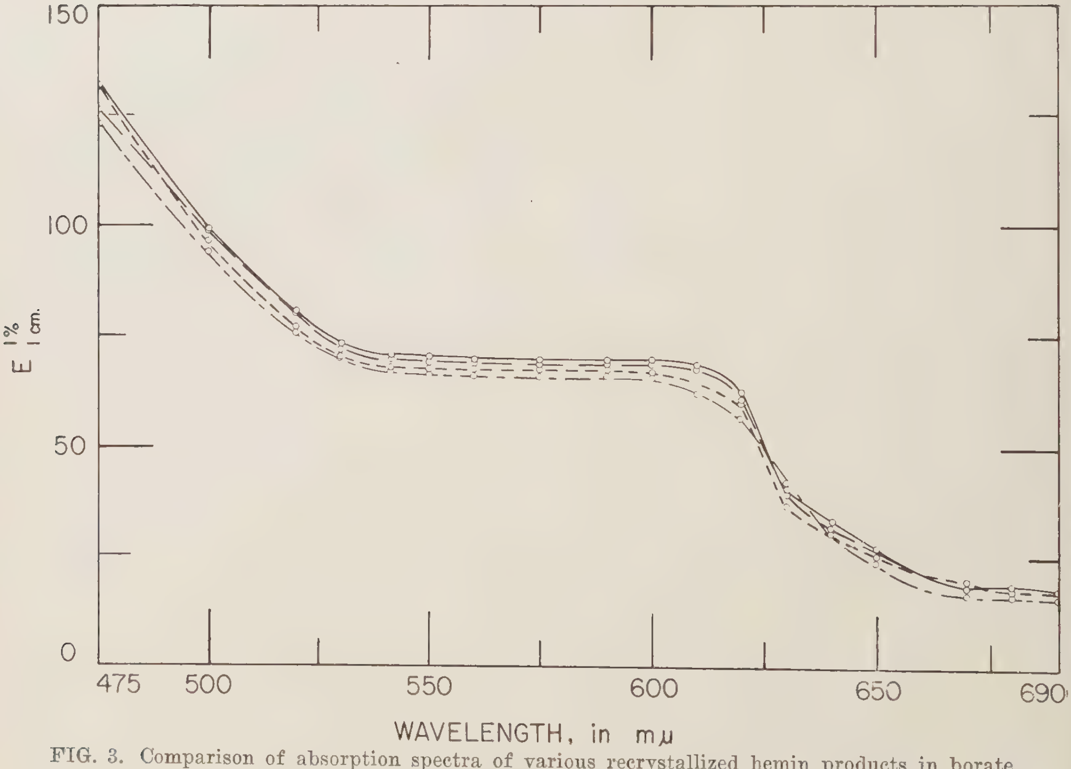

FIG. 3. Comparison of absorption spectra of various recrystallized hemin products in borate buffer.

shown in Fig. 2, and the same relationships for the same preparations after one recrystallization are presented in Fig. 3. Since replicate values for the various dilutions of each product were in excellent agreement, average values are plotted, each curve representing

TABLE II. Ratio of Extinction Coefficients of Dilute Solutions of Crude and of Recrystallized Hemin at 580 mμ over a Period of One Month.

Sample	Ratios of $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\ cent}}$ at 580 mμ	
	16 days	1 mo
Crude crystalline bovine hemin (authors)	1.066	1.108
1st recrystallization bovine hemin (authors)	1.015	1.015
2nd	1	1
3rd	1	1
4th	1.014	1.014
Crude amorphous hemin (Armour lab.)	1	1
Recrystallized hemin (Armour lab.)	1.015	1.029
Crude crystalline human hemin (authors)	1.014	1
Recrystallized human hemin (authors)	1.015	1.015

the mean extinction coefficient for 3 concentrations of each preparation. It is evident that recrystallization of hemin makes for significantly greater uniformity and reproducibility among several preparations. Another way to show this effect of recrystallization is by calculating the ratio of the highest extinction coefficient at a particular wavelength to the lowest at the same wavelength. For the crude hemin preparations this ratio at 590 mμ is 1.36; for the recrystallized material it is 1.05, indicating a much smaller variation in the degree of light absorption by the recrystallized hemin.

Ratios of extinction coefficients are also used to present the effect of age of hemin solutions on their stabilities. In Table II are presented the ratios of $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\ cent}}$ for dilute solutions of the several hemin preparations 16 and 30 days after dilution to the values of $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\ cent}}$ of the same solutions immediately after being diluted. All ratios are calculated at 580 mμ and represent the maximum deviations of the aged solutions from the fresh dilutions. It is evident that the recrystallized samples were more consistently stable than the crude preparations.

Similar studies on the stock solutions indicated that concentrated solutions of hemin are stable regardless of purity, as the ratios of $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\ cent}}$ were very close to unity for all samples.

Summary. 1. The purity of crude hemin preparations used, as determined by their iron content, was improved by recrystallization. 2. Crude hemin preparations in borate buffer showed variable extinction coefficients at any wavelength selected along the flat portions of the absorption spectra. Recrystallized hemin preparations from the above crude hemin samples showed very nearly equal extinction coefficients at the same wavelength. 3. Dilute solutions of recrystallized hemin from different sources were more uniformly stable than dilute solutions of crude hemin during a one-month period.

The authors wish to express their appreciation to Armour Laboratories for the hemin samples given for this study.

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Experimental Arteriosclerosis: Inhibitory Effects of Ascorbic Acid and Inositol. (19850)

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The term arteriosclerosis has been variously defined. Too commonly, lipid, and in particular cholesterol infiltration (atheroma), has been identified as the essential lesion in this syndrome. For purposes of this paper we have defined arteriosclerosis as any thickening, or increase in density, of the arterial wall. Atherosclerosis may be defined as a degenerative metabolic subdivision of arteriosclerosis, characterized by hyaline degeneration, lipid infiltration and finally, calcinotic changes of the intima and/or media.

Our immediate purpose in investigating this problem, was 2-fold: 1. To produce experimentally, in the rabbit, arteriosclerotic lesions of the aorta. 2. To inhibit or reverse the formation of these lesions. Josue(1) observed experimentally, that repeated injections of epinephrine were capable of producing sclerosis of the aorta. Numerous investigators(2,3) have, since that time, reaffirmed this experimental fact, usually describing the resultant plaques as erosions or necroses of the tunica media. Oester and Mikulicich(4) have recently observed that combined injections of epinephrine and thyroxine result in an apparent potentiation in the production of this experimental aortic sclerosis. The present communication is a report on the results of experiments with inositol and ascorbic acid, used separately, in an endeavor to influence the epinephrine-thyroxine induced arteriosclerosis in rabbits.

Methods. Rabbits totalling 111, were used. The method for production of the sclerosis was as follows. Twenty-five μg of epinephrine hydrochloride per kilo of body weight were very slowly injected intravenously by ear, on 2 consectutive days. On the third day the dose of epinephrine injected was increased to 40 μg per kilo. On the fourth day it was increased to 50 μg per kilo. This regimen of 50 μg of epinephrine per kilo of body weight was maintained for 11 more days, a total of 15 days of epinephrine administration. Thyroxine was injected subcutaneously, in the region of the middle of the back, daily, for the 15 days, at a constant dose level of 0.15 mg/kg body weight. Post-mortem examinations were routinely made. Our primary observations were concerned with the frequency and severity of sclerosis in the aorta from the region of the heart into the iliac bifurcation. Final conclusion as to whether sclerosis was actually present was based on histological study. It is virtually impossible to compare plaques accurately, but for purposes of differentiation we undertook their gradation on the following basis: zero, no plaques; 1, mild single patches (plaques); 2, mild, scattered patches; 3, severe, extensive patches; 4, widespread, confluent and extensive patches. The control group of 25 animals received only epinephrine and thyroxine injections. Identical regimens of epinephrine and thyroxine injections were also administered to the groups of animals which received inositol, and to those which received ascorbic acid. Thirty-six animals were treated with ascorbic acid, 18 received 100 mg per day, 9 received 500 mg per day, and 9 received 50 mg per day. All ascorbic acid was administered subcutaneously in the region of the middle of the back. The ascorbic acid treatment was begun 3 days before epinephrine and thyroxine. All injections were continued for 15 more days, thereby totalling 18. Thirty animals were treated with inositol, the subcutaneous injections being given, as with Vit. C, before and during the epinephrine and thyroxine for a total 18 day program. The animals were divided by dose of inositol into 2 groups of 15 each, the first receiving 3 g per kilo per day, the second 6 g per kilo per day. Table I provides a summary of the protocol from these experiments. Table II is an analysis of the data from Table I.

Results and discussion. Investigation with massive doses of inositol indicates it has no significant value in the treatment or prophylaxis of the experimental epinephrine-

TABLE I. Influence of Inositol and Ascorbic Acid on Epinephrine-Thyroxine Sclerosis.

Treatment*	No. of rabbits	Deaths before 6 days	Deaths 6 to 15 days	No. animals sacrificed on 16th day	Degree of sclerosis				
					0	1	2	3	4
E + T (C)	25	5	12	8	2	4	4	3	7
C + I, 3 g/kg	17	2	12	3	3	5	3	3	1
" 6 "	19	4	11	4	4	4	3	4	0
C + A, 50 mg/kg	12	3	5	4	3	2	1	1	2
" 100 "	22	4	11	7	7	2	4	3	2
" 500 "	10	1	6	3	4	1	2	2	0

* E = Epinephrine; T = Thyroxine; C = Controls; I = Inositol; A = Ascorbic acid.

TABLE II.
Significance of Influence of Inositol and Ascorbic Acid on Epinephrine-Thyroxine Sclerosis.

	No. with sclerosis /Total No. animals	% incidence	Probability*	Severity factor (% incidence × avg degree of sclerosis)
E + T (C)†	18/20	90		90 × 2.45 = 220
C + I, 3 g/kg	12/15	80	>.1	80 × 1.6 = 128
" 6 "	11/15	73	>.1	73 × 1.46 = 106.5
C + A, 50 mg/kg	6/9	66	>.2	66 × 1.66 = 109.5
" 100 "	11/18	61	<.01	61 × 1.5 = 91.5
" 500 "	5/9	55	>.1	55 × 1.22 = 67.1

* Probability compared to controls, a value of <.05 is assumed significant. Probability: (1) for total inositol group = >.1; (2) for total ascorbic acid group = <.01. Animals whose death occurred before 6th day of treatment were not included in biometrical analysis.

† E = Epinephrine; T = Thyroxine; C = Controls; I = Inositol; A = Ascorbic acid..

thyroxine arteriosclerosis. Ascorbic acid proved valuable in the inhibition of these lesions, especially in daily doses of 100 and 500 mg. The tables indicate the results which were obtained. The data obtained with the use of inositol, although it demonstrates a lower incidence of sclerosis, is not of statistical significance. The results with ascorbic acid are of statistical significance. In addition, the severity of the sclerosis developed under the influence of ascorbic acid was markedly less than either the controls or when inositol was used. This is shown by the lessened number of cases which were recorded as four plus sclerosis. In addition, a factor which we call severity factor also expresses this relationship. This number is obtained by multiplying the percentage incidence of sclerosis in any group by the average degree of sclerosis, expressed in our plus units. The higher this number, the higher is the total gross degree of sclerotic severity. It is seen that this value is markedly lower with each of the ascorbic acid groups and there is a progressive decrease with increasing dose of ascorbic acid.

The severe median and often intimal sclerosis induced in the aorta of rabbits by combined injections of epinephrine and thyroxine indicates the possibility that such a combination of hormonal influences may be active in the production of arteriosclerosis in other animals.

Epinephrine scleroisis, potentiated by thyroxine and perhaps by other hormonal imbalances, may exert sufficient injury to the arterial wall so that later, lipid deposition is favored. The experimentally demonstrable influence of epinephrine and thyroxine might be considered as part of the pre-lipid phase of atherosclerosis. It is our feeling that some shock phase precedes active lipid-infiltration. We suggest that a dysfunction of lipid metabolism may be a factor which is secondary to the lesions resulting from these shocking stresses, hormonal or otherwise.

The role of thyroxine in the production of arteriosclerosis is paradoxical and, as yet, not well defined. It has been used successfully to produce remission of the experimental cholesterol sclerosis(5) and yet as stated it adds significantly to the degree and incidence of the sclerosis produced by epinephrine in the rabbit. This last fact is supported somewhat by the findings of Lortat and Sabareaunu(6) who inhibited the epinephrine-induced sclerosis by extirpation of the thyroid gland.

Summary. 1. Combined injections of epinephrine and thyroxine were used to produce, in rabbits, aortic sclerosis which was characterized by lack of atheroma, and the presence of severe medial and intimal sclerosis. 2. 90% of all the control animals developed aortic sclerosis when treated with epinephrine and thyroxine. 3. Massive doses of inositol appeared to inhibit the influence and severity of these lesions, to only a slight degree. 4. Daily concomitant administration of 50 mg of ascorbic acid lowered the incidence of scleroses to 66%; 100 mg lowered the incidence to 61%; 500 mg lowered it to 55%. The severity of the lesions produced was also considerably lessened. The inhibition due to ascorbic acid was statistically significant.

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Effect of Bacteria and Their Products on Migration of Leukocytes.* (19851)

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In the course of studies of a purified toxic somatic antigen from *Salmonella typhosa*, Morgan(1) noted that leucocytes from guinea pig splenic explants failed to migrate towards a capillary tube containing this material. The technic used in this test was that of Chambers and Legrand(2) which consisted of placing the bacterial product in a capillary tube adjacent to the explant and noting the direction of migration of the leucocytes. The failure to migrate toward the typhoid somatic carbohydrate was interpreted as negative chemotaxis. When whole typhoid bacilli were employed, slight positive chemotaxis was noted. It was reported by Delauney and Pages(3) that the injection of the "endotoxins" of *Salmonella typhosa* prevented the diapedesis of leucocytes from the blood vessels of animals. Miles and Nivens(4) confirmed these findings and believed that this occurred because of poor tissue perfusion secondary to the circulatory failure produced by the endotoxins. The present study deals with the effect of a series of bacteria and some bacterial products on leucocytes. The tests were carried out in a special cell(5) which permits quantitation of the capacity of leucocytes to migrate from a buffy coat culture.

Methods and material. Human blood was used, silicone (GE No. 9986) treated glassware, syringes, and needles were employed throughout; heparin in final dilution of 1 μg per ml of blood was used to delay clotting. The organisms or purified bacterial products were added to the blood in quantities outlined in the tables. The bacterial products were suspended in isotonic saline and the final dilution of one part of the material to 9 parts of blood was used. The slide cell for centrifugation has been modified by the use of 2 lucite blocks held together by screws and separated by a rubber band (Fig. 1). Cells were filled to the midway mark with blood and the bolts

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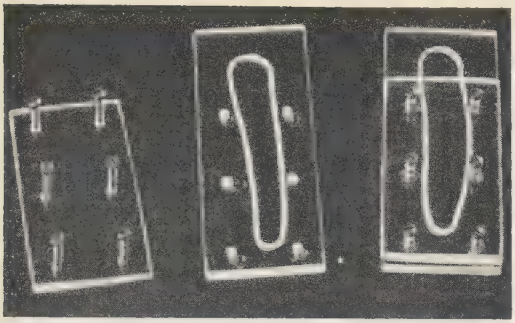

FIG. 1. Photograph of slide cell. The cell on the right is completely assembled while the two parts are shown on the left.

were tightened until the plates were approximately 0.25 mm apart. The cells were sealed with petrolatum. Centrifugation of the cells at 200 G. for 5 minutes gave adequate separation of plasma, white cells, and red cells. After incubation at 37°C for 5-15 minutes the plasma clotted. The slide cells were removed and examined at intervals under the microscope. The whole organisms tested were *S. typhosa* and *Ps. aeruginosa* from Dr. D. T. Smith and *D. pneumoniae* type I from Dr. M. McCarty. The organisms were grown on agar surface and diluted as necessary. The purified material included meningococcal filtrate from Dr. B. Black-Schaffer, typhoid somatic antigen from Dr. H. R. Morgan, Piromen from *Ps. aeruginosa* from Dr. L. Ginger of Baxter Co.; P25 polysaccharide from *Serratia marcescens* from Dr. M. J. Shear and type-specific polysaccharide from type III pneumococcus from Dr. O. T. Avery. The *M. aureus*, coagulase positive, was obtained from a patient with bacteremia.

Experimental results. The results presented in Table I indicated the effect of various bacteria on the oriented migration of human leucocytes. *S. typhosa* and *P. aeruginosa* in concentrations of 6 × 10^6 organisms per ml produced inhibition of migration. *M. aureus* and *D. pneumoniae* in concentrations of 6 × 10^8 organisms per ml produced no inhibition of migration.

Table II outlines the effect of various bacterial products on the migration of leucocytes. There is a striking difference between the effect of the polysaccharide of the pneumococcus and the other bacterial products. The leucocytes start migrating at a normal rate in all tests but within 1½ to 2 hours the leucocytes exposed to material from *S. typhosa, Ser. marcescens, P. aeruginosa* and filtrate of the *N. intracellularis* show marked slowing. Table II shows wide variation in the concentration necessary to inhibit the migration. This is due to the marked variation from individual to individual in the level required to produce inhibition. As the concentration is decreased, a zone of stimulation is reached. This is manifested by greater rate and distance of migration. It should be mentioned that these substances, like the *M. tuberculosis,* cause clumping of leucocytes in the slide cell.

Discussion. Inhibition of the migration of leucocytes is produced by a group of bacteria and their soluble products. This inhibition is in contrast to the lack of effect of another group of bacteria and their products. The organisms producing inhibition are characterized by the production of endotoxins. These endotoxins have certain characteristic effects noted when given intravenously to animals. It would seem that the leucopenia and fever noted *in vivo* may be related to the *in vitro* effect reported here. The direct effect of this material on leucocytes as well as the shock may contribute to the altered inflammatory response noted by previous workers when some of these materials are given intraven-

TABLE I. Effect of Whole Bacteria on Migration of Leucocytes.

		Org./ml
S. typhosa	Inhibition	6 × 10^6
P. aeruginosa		
M. aureus	No inhibition	6 × 10^8
D. pneumoniae Type III		

TABLE II. Effect of Purified Bacterial Products on Migration of Leucocytes.

		γ/ml
Typhoid somatic carbohydrate (Morgan)	Inhibition	.058– .58
P. aeruginosa carbohydrate (Baxter Piromen)		.25 –5
Meningococcal filtrate		.005– .01
Polysaccharide from Serratia (Shear)		.036– .36
Polysaccharide pneumococcus Type III		2000

ously(3,4,6). Sarcoma 37 in contrast to the leucocyte does not behave in the same manner toward the material from Serratia since there is no demonstrable effect in tissue culture (7,8).

The inhibition by the material from Gram negative organisms would appear to be more complex than "negative chemotaxis" since the material is distributed throughout the medium of red cells, white cells and plasma. The zone of stimulation noted with decreasing concentrations of the material would be more in favor of a direct effect on the leucocyte.

Summary. The complex carbohydrate from *S. typhosa, P. aeruginosa,* and Serratia as well as a filtrate from *N. intercellularis* inhibited the migration of human leucocytes. The same effect was noted with whole bacteria of *S. typhosa* and *P. aeruginosa.* No inhibition was seen with *M. aureus, D. pneumoniae* Type III or with the polysaccharide of Type III.

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Role of Pancreas in Hypoglycemia Responsiveness and Associated Rise of Circulating Eosinophiles.* (19852)

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In the normal organism the intravenous administration of a small dose of crystalline insulin (0.1 unit/kilo) causes an abrupt decline of the blood sugar with the nadir at 20 to 30 minutes followed by a sharp secondary rise reaching the fasting level by 150 minutes (1). This secondary rise is probably not due to a termination of the insulin effect, since in experiments in which the duration of action of insulin is measured by the time during which glucose must be injected in order to prevent the blood sugar level from declining, it has been found that 5 units of insulin have a duration of action of 6 hours(2). This evidence, together with the fact that hypophysectomized and adrenalectomized animals in which the apparent duration of insulin action is much greater than in the normal, has led to the hypothesis that the secondary rise of the blood sugar level in the insulin tolerance test is a result of stimulation of a body homeostatic mechanism which tends to restore the original blood sugar level(3,4). This phenomenon is properly termed hypoglycemia responsiveness and in order for it to make its appearance two conditions must be met: (1) There must be a sufficient reserve of hepatic glycogen to allow for the observed rise in the blood sugar concentration and (2) a mechanism for glycogenolysis must be activated by the falling blood sugar level(5). It has been hypothecated that the mechanisms stimulated by hypoglycemia are located in the hypophysis, the adrenal cortex, or the sympathetic nervous system(3,4). In a previous study it was observed that insulin administered to normal individuals is accompanied by a marked rise of the absolute lymphocyte count(6). In diabetic patients, however, the extent of the

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rise was proportional to the degree of hypoglycemia responsiveness evidenced by the insulin tolerance test(7). This led to the hypothesis that the trigger mechanism for hypoglycemia responsiveness is identical with that causing an increase in the absolute lymphocyte count after insulin administration. Similarly, it was also found that the eosinophile level parallels the change in absolute lymphocyte count(8,9,10,11). The result of experimental studies undertaken for the purpose of localizing the site of this trigger mechanism may be summarized as follows: 1) In previous studies it was shown that the eosinophile(11) and the blood sugar response(12) of the adreno-demedullated dog to insulin are similar to those of the intact animal. 2) Both hypophysectomized and adrenalectomized animals demonstrate an impairment of hypoglycemia responsiveness but a rise of the eosinophile level after insulin(11). This impairment of hypoglycemia responsiveness, however, is probably attributable to the depletion of liver glycogen in these animals rather than the ablation of the trigger mechanism. 3) In growth hormone treated adrenalectomized animals the response to insulin hypoglycemia is restored to normal(13). These data would tend to rule out the hypophysis, the adrenal cortex or the adrenal medulla as the seat of this mechanism.

The present study was undertaken to determine whether the pancreatic hyperglycemic factor may be implicated in the dual mechanism of hypoglycemia responsiveness and the associated rise of the eosinophile level.

Material and methods. The study was carried out on four groups of dogs: 1) 10 intact control animals who received 0.1 unit of crystalline insulin (Lilly) per kilo of body weight intravenously. 2) 5 intact animals who received insulin free of hyperglycemic factor (Lilly Lot No. T-2536) instead of crystalline insulin. 3) 5 intact animals who received 1 cc of normal saline solution intravenously instead of insulin. 4) 5 pancreatectomized dogs who received 0.1 unit of crystalline insulin per kilo of body weight intravenously. Duplicate experiments were done in this group of animals with insulin devoid of hyperglycemic factor.

All animals had been maintained on a diet of canned dog food and table scraps. In addition, the pancreatectomized animals were given 5 g of lecithin and 12 units of regular insulin twice daily. No insulin, however, was administered on the day preceding the experiment. Furthermore, the experiments in operated animals were not carried out until the 14th postoperative day and were repeated in the same dog at 2 week intervals. The test material was injected after a 24 hour fast and 2 hours after the induction of intraperitoneal nembutal anaesthesia. When necessary as indicated by a returning corneal reflex, the animals were given additional small doses of anaesthetic. Duplicate venous blood samples for sugar and direct eosinophile counts were collected at 0, 20, 30, 45, 60, 90, 120, and 150 minutes after the injection. The blood sugar determinations were done by the Nelson modification of the Folin-Wu micro method(14) and the eosinophile counts were done according to the procedure of Randolph (15). In conformity with the method of reporting results used previously(16,17,18) the change of the eosinophile level was expressed in terms of the percentage change from the fasting value which was taken as zero percent.

Results. In the 80 determinations of the absolute eosinophile level in these 10 controls, the values ranged from +22% to —16% of the initial value with a standard deviation of ±8%. Therefore, in evaluating the experimental series, any change of the absolute eosinophile level of ±24% (3 times the standard deviation) of the fasting value or less was considered to be non-significant.

The experiments were started two hours after the induction of anaesthesia since it was found that in the untreated normal dog this time interval allowed for recovery from the initial stress incident to anaesthetizing the animal.

Results. The mean variation of the blood sugar and the eosinophile level of the saline control animals are represented in Fig. 1, Curve A and A′.

After the administration of commercial crystalline insulin to the intact animal the lowest blood sugar level was reached at 20,

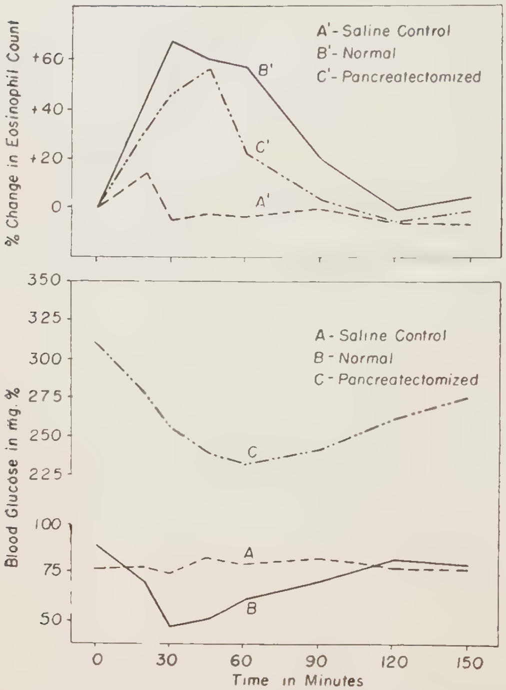

FIG. 1. Mean blood sugar levels and mean percentage change of the eosinophile count after the intrav. inj. of insulin in normal and pancreatectomized dogs compared with saline controls.

30, or 45 minutes whence it rose gradually again approaching the fasting level by 150 minutes. This was accompanied in all instances by a significant rise in the eosinophile level varying from +45% to +120% of the fasting value which reached its peak at 30 to 60 minutes and was then followed by a gradual decline. The change in the blood sugar and eosinophile level after insulin free of hyperglycemic factor was in all respects similar to that observed after crystalline insulin. The mean values both of blood sugar and eosinophiles are shown in Fig. 1, Curve B and B').

In the pancreatectomized animal the mean fasting blood sugar level was 315 mg %. After the administration of insulin the lowest mean blood sugar of 230 mg % was reached at 60 minutes and it then increased gradually so that the mean 150 minute blood sugar value was 45 mg % above nadir (Fig. 1, Curve C). The accompanying change in the eosinophile level was almost the same as in the intact animal except that the peak response was moderately delayed and somewhat lower (Curve C'), the value varying from +48% to +87% of the fasting level.

Comment. These results indicate that the presence of the pancreas is not necessary for the hypoglycemia responsiveness after the injection of insulin nor for the associated rise of the eosinophiles.

That this rise of eosinophile level is probably not due to a foreign protein reaction is shown by the fact that inactivated insulin when similarly injected is not accompanied by this phenomenon.

The principal evidence for the role of hyperglycemic factor as a physiologic insulin antagonist has been the finding of an impurity in ordinary crystalline insulin which raises the blood sugar level and which can be isolated from the pancreas even in alloxan diabetic animals(19). This substance has been shown to act through hepatic glycogenolysis(20). The relatively more severe diabetes of the alloxan diabetic as compared to that of the pancreatectomized dog has also been attributed to this substance(21).

It is possible that hypoglycemia responsiveness is effected by a direct action of the glycogenolytic fibers of the sympathetic system on the liver and that the rise of the eosinophile level after insulin may also be due to sympathetic stimulation. This possibility is supported by the fact that stimulation of the nerves of the hepatic plexus will produce hyperglycemia in the fed adrenalectomized animal by breakdown of liver glycogen, and that the sympathectomized animal has an increased insulin sensitivity(22). Studies to test this hypothesis are in progress.

Summary. In the pancreatectomized animal the intravenous administration of both commercial crystalline insulin and insulin free of hyperglycemic factor is followed by a rise of the absolute eosinophile count and associated decline of the blood sugar level with a subsequent rise, similar to that observed in the normal. This is interpreted to signify that the hyperglycemic factor of the pancreas is not part of the mechanism for hypoglycemia responsiveness.

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Effects of Shock and Cold on Mouse Liver Sulfhydryl.* (19853)

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While testing the effects of *S. marcescens* tumor damaging bacterial polysaccharide(2) on concentration of iodine-reducing substances in mouse tissues, one of us noted(1) that toxic doses of this substance induced in mouse liver an appreciable decrease in concentration of non-protein sulfhydryl, measured as the difference between total iodine-reducing substances and iodine reduction due to ascorbic acid. Toxic doses of the polysaccharide markedly depress body temperature, circulation and reactivity of mice(3,4,5), as do also burns(6) and ligation trauma(7). This suggested the desirability of testing for effects of burn shock and traumatic shock on concentration of sulfhydryl compounds in mouse liver. The effects of a non-shock stress situation (exposure to cold) on liver sulfhydryl were also studied.

Materials and methods. Distilled water used: Ordinary distilled water was passed through a LaMotte Filter-ion exchange column, to remove traces of heavy metal ions reactive with sulfhydryl groupings. *Preparation of liver extracts.* A. *Solutions.* M/10 potassium phosphate buffer of pH = 6.8 (KH_2PO_4, K_2HPO_4 1:1); 22% aqueous sulfosalicylic acid. B. *Procedure.* The test mouse was decapitated, the liver promptly excised, blotted to remove excess blood, weighed to within 10 mg, placed in the micro-cup of the Waring blender, and homogenized with 10 cc of ice-cold phosphate buffer for 30 seconds. Additional phosphate buffer was then added, to a total of 19 cc of the buffer for each gram of liver tissue. Homogenization was continued for another 30 seconds. The homogenate was centrifuged at about 500 g for 5 minutes to

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* We are indebted to Merck & Co., Rahway, for a Grant-in-Aid of this research, and to Dr. M. J. Shear, National Cancer Inst., for the sample of *S. marcescens* polysaccharide used.

give a supernatant (Extract 1). A 10 cc aliquot of Extract 1 was added to 1 cc of 22% sulfosalicylic acid, and the mixture shaken vigorously, and poured through Whatman No. 1 paper to give a protein-free filtrate (Extract 2). *Amperometric titrations for sulfhydryl concentrations in Extracts 1 and 2*: Except that a vibrating platinum electrode was substituted for the revolving platinum electrode, these titrations were carried out essentially as described by Weissman, Schoenbach and Armistead(8). Diluted methanol, prepared as described by them, was used in all titrations, including those of glutathione standards. Titration of 2 or 4 cc of Extract 1 was started as soon as possible after the centrifuging, to give a value for total sulfhydryl extractable from mouse liver (concentration in liver taken as 20 times that in Extract 1). Five cc of Extract 2 were then titrated, to give a value for non-protein sulfhydryl extractable from mouse liver (concentration in liver taken as 22 times that in Extract 2). Protein sulfhydryl has been estimated as the difference between total and non-protein sulfhydryl. *Protein concentration in Extract 1*: This was estimated by the Biuret method of Robinson and Hogden(9), using a Beckman spectrophotometer set at 560 mμ for the optical density readings. In each case, 2 cc of Extract 1, 8 cc of 3.75% NaOH and 0.25 cc of 20% $CuSO_4 \cdot 5H_2O$ were used. Biuret optical density readings were standardized against casein standards, and against dry weights of material, presumably protein, which was precipitated from Extract 1 by 22% sulfosalicylic acid. *Mice used:* CAF_1 male mice were secured from Jackson Laboratories, Bar Harbor, Maine, and were used when weighing 22-27 g. ABC female mice were secured from Dr. J. J. Bittner, University of Minnesota, Minneapolis, Minn., and used when weighing 26-30 g. *Scalding of mice:* The procedure for inducing burn shock described by Rosenthal(6) was employed. The test mouse was etherized and dipped to the neck in water at 60°C for 10 seconds, or in water at 65°C for 15 seconds. Three, 6 or 18 hours after scalding, the mouse was sacrificed, and liver extracts obtained as described above. Unscalded mice were used as controls. Some of these were untreated, some had been etherized and dipped to the neck at 37°C for 10 or for 15 seconds. *Production of ligation trauma:* The procedure described by Rosenthal(7) for production of traumatic shock was employed. The time between placement of the rubber bands around both hind legs and their removal was set at 2 hours. Livers were secured from test mice 2 hours after removal of bands. *Administration of S. marcescens polysaccharide:* A water solution of polysaccharide(2), preparation P25, was injected intraperitoneally in the amount of .01 cc per gram, to give a dose of 16 mg per kg. Controls were injected with distilled water. The dose of polysaccharide was appreciably greater than the minimum of 2 mg or less per kg found necessary to induce marked hemorrhagic necrosis in mouse Sarcoma 37, implanted not less than 6 days earlier, and somewhat less than the minimum dose at which deaths were encountered. Eighteen hours after injection of polysaccharide or water, the mice were sacrificed and livers excised for sulfhydryl determinations. *Exposure to cold:* Two mice were simultaneously placed in a round glass container having a surface area at the bottom of about 200 sq. cm. The container was placed in a refrigerator just beneath the freezing compartment. Air in the glass container near the mice had a temperature between 5° and 10°C. The mice were sacrificed for liver sulfhydryl determinations 5 hours after having been placed in the refrigerator.

Results: Mice were in shock or a shock-like state at 3 and at 6 hours after scalding, at 2 hours after removal of ligatures from both hind legs, and at 18 hours after injection of polysaccharide. All of these mice were lethargic, indifferent to stimulation, cold to the touch and dyspneic. Mice which had been scalded to the neck at 60°C for 10 seconds, 18 hours earlier, or which had been exposed to cold for 5 hours, were obviously not in shock. They were fairly active, responsive to stimulation and not dyspneic. Although these mice felt warm when held in the hand, those which had been exposed to cold exhibited rectal temperatures which were 1-2°C lower than those exhibited before exposure to

TABLE I. Effects of Scalding on Mouse Liver Sulfhydryl (–SH).

Group No.	Mice used			Treatment	Hr between treatment and sacrifice	Liver –SH (GSH equiv., in mg % ± stand. error)	
	Strain	Sex	No.			Non-protein –SH	Protein –SH
1	CAF$_1$	♂	7	C:* no treatment	—	210 ± 17	511 ± 19
2			8	C: dipped at 37°C, 10″	3	197 ± 18	505 ± 30
3			7	S* at 60°C, 10″	3	116 ± 11	534 ± 23
4			8	C: dipped at 37°C, 15″	3	201 ± 23	588 ± 24
5			11	S at 65°C, 15″	3	121 ± 10	522 ± 16
6			10	C: no treatment	—	201 ± 7	479 ± 18
7			10	S at 60°C, 10″	18	260 ± 17	426 ± 23
8	ABC	♀	10	C: dipped at 37°C, 10″	3	207 ± 11	486 ± 13
9			10	S at 60°C, 10″	3	141 ± 18	544 ± 22
10			10		6	124 ± 13	470 ± 19
11			10		18	281 ± 10	474 ± 30

* C = Controls; S = Scalded.

TABLE II. Effects of Ligation Trauma, *S. marcescens* Polysaccharide and Exposure to Cold on Mouse Liver Sulfhydryl (–SH).

Group No.	Mice used			Treatment	Hr between treatment and sacrifice	Liver –SH (GSH equiv., in mg % ± stand. error)	
	Strain	Sex	No.			Non-protein –SH	Protein –SH
1	CAF$_1$	♂	10	C:* no treatment	—	239 ± 11	560 ± 23
2			11	Hind legs ligated, 2 hr	2	112 ± 14	500 ± 13
3			10	*S. marcescens* polysaccharide, IP, 16 mg/kg	18	106 ± 13	448 ± 19
4*			10	C: no treatment	—	201 ± 7	479 ± 18
5			10	At 5-10°C, 5 hr	0	121 ± 14	433 ± 16

* C = Controls.

cold. ABC female mice were apparently in better shape at 18 hours after scalding than were CAF$_1$ male mice. *Liver sulfhydryl:* Table I, Groups 3, 5, 9 and 10, show that average values for liver *non-protein sulfhydryl* concentration secured for mice apparently in shock, and sacrificed 3 or 6 hours after scalding, were significantly less than those exhibited by mice of corresponding control Groups 1, 2, 4 and 8. (P values less than .01). However, groups of mice recovering from shock, at 18 hours after scalding (Table I, Groups 7 and 11), exhibited average liver *non-protein sulfhydryl* concentrations which were actually significantly greater than those exhibited by corresponding control groups 6 and 8 (P values less than .01). Table II indicates that statistically significant decreases in average values for liver *non-protein sulfhydryl* concentration were also exhibited by mice which had been subjected to: a) ligation trauma (Group 2), b) a toxic dose of *S. marcescens* polysaccharide (Group 3), and exposure to cold (Group 5) (all P values less than .01). Some groups of the experimentally treated mice (Table I, Groups 5 and 7; Table II, Groups 3 and 5) also exhibited moderate but apparently significant decreases in liver *protein sulfhydryl* concentration. The mice of Table I, Group 7, exhibited at 18 hours after scalding an increase in liver *non-protein sulfhydryl* and a *decrease* in liver *protein sulfhydryl* concentration. *Protein extracted from liver with phosphate buffer:* The amount of protein present in Solutions 1, as indicated by Biuret optical density readings and dry weights of material precipitated from Solutions 1 by sulfosalicylic acid, was about 2-2.5%, indicating the presence in mouse liver of extractable protein in the amount of 20-25% of the wet weight of the liver. Values for control and treated mice have not differed significantly, hence the data for individual Groups have been omitted.

Discussion. The work of Binkley and coworkers(10) has indicated that the non-protein sulfhydryl of rat liver consists mainly of glutathione or precursors of glutathione, *i.e.*, cysteinyl glycine and y-glutamyl cysteine. We have no data as to the nature and amounts of compounds which comprise the non-protein sylfhydryl of mouse liver, nor which ones are concerned in the observed changes in non-protein sulfhydryl occurring as a result of the experimental procedures to which the mice have been subjected. Changes in non-protein sulfhydryl observed in the present experiments probably were due chiefly to changes in relative rates of synthesis and breakdown of glutathione in the liver. Further experimentation may reveal whether decreases in liver non-protein sulfhydryl concentration are a usual concomitant of stress situations and shock, and what mechanisms are involved in the effects noted to date.

Summary. Mice were subjected to severe stress by scalding, ligation trauma, injection of a toxic dose of a bacterial polysaccharide, or exposure to cold. Each of these procedures produced a marked decrease in liver non-protein sulfhydryl concentration. In some experiments, a moderate decrease in liver protein sulfhydryl concentration also occurred. Mice recovering from scalding exhibited marked increases in liver non-protein sulfhydryl concentration to values well above the original control level.

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An Inhibiting Effect Observed in the Course of Normal Wound Healing.
(19854)

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The experiments described herein were initiated to attempt to account for the slowing-down of the proliferation of granulation-tissue in the last phase of wound healing prior to closure by epithelization. One possibility is that this slowing-down of the proliferation is caused by the liberation of an inhibiting factor in the organism.

To test this hypothesis, 2 experimental wounds of equal size were produced at different time intervals, so that any inhibiting influence produced in the last phase of the first wound healing process could be made to coincide with the active phase of proliferation of the granulation-tissue in the second wound.

Material and methods. Four series of experiments were performed on male albino rats of about 200 g weight. 1) A circular skin wound, 20 mm in diameter, was produced after depilation on the back of the animal on one side of the dorsal midline, and a second wound of equal size added symmetrically on the other side of the dorsal midline after an interval of 6-7 days (16 animals). The healing wounds were outlined on every second day and the values thus obtained plotted in curves. The exact technic of wound production and measurement has been described

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Exp.	2 wounds separated by:		Mean healing times ± S.D.* (days)	Diff. of mean healing times ± S.D.* (days)
	days			
1	6–7	1st wound	26.4 ± 1.1	12 ± 2.8
		2nd	38.4 ± 2.6	
2	10	1st	25.9 ± 1.1	1.9 ± 1.5
		2nd	27.8 ± 1	
3	14	1st	26.8 ± 1.7	3.8 ± 2.6
		2nd	30.6 ± 1.9	
4	18–23	1st	26.9 ± 1	3.7 ± 1.9
		2nd	30.6 ± 1	
Control exp.				
1	2 wounds produced simultaneously on 1 animal:	Right wound	22.5 ± .7	.6 ± 1.1
		Left	21.9 ± .9	
2	1 single wound:		21.6 ± 1.1	

* Stand. dev. of the mean.

(1,2). In order to assess the time during which the assumed inhibitory substance was effective, 3 other sets of experiments under the same conditions were undertaken, producing 2 equal wounds in one animal. 2) The second wound 10 days after the first one (8 animals). 3) The second wound 14 days after the first one (8 animals). 4) In the fourth experiment the second wound was produced 3-6 days before the first wound closed, *i.e.,* 18-23 days after the wound had been produced (14 animals). Control experiments with one single wound (36 animals) and with 2 equal wounds produced simultaneously in one animal (15 animals) were made so as to obtain the normal curve of the wound healing process. If a marked difference between the size of the 2 wounds occurred unintentionally, these experimental animals were discarded, as mathematical evaluation of all experiments clearly showed that the greater the difference between the initial wound surfaces the smaller became the difference of the respective healing times. These results are in accordance with those of Carrel and Hartmann(3). In case of infection the experiment was also discarded.

Results: (See tabulation above).

In Exp. 1 in which the second wound was produced 6-7 days after the first wound, a marked inhibition of the wound healing process of the second wound appeared during the last 7-10 days before the first wound was closed, *i.e.,* 15-20 days after the production of the first wound. While the healing process of the first wound ran its normal course(2) to the very end, the second wound for several days showed a diminished tendency to heal, so that the healing process was completed only after a considerable delay of 12 days. (See Fig. 1, one of the characteristic curves). The healing time of the second wound was also considerably extended as compared with the control experiments with one single wound (21.6 days) and with 2 wounds produced simultaneously in one animal (22.2 days).

The other 3 experimental series (2-4) showed only a short inhibition in the healing of the second wound.

It should be noted that the healing of the first wound in Exp. 1 to 4 was also delayed in comparison with the controls.

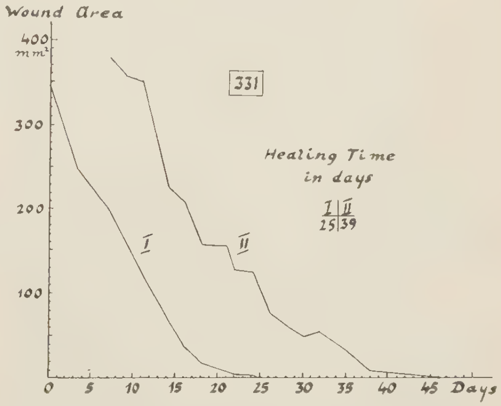

FIG. 1. Healing curves of 2 wounds produced 7 days apart, demonstrating inhibition of healing in the second wound during the last phase of the first.

The weight of the animals remained approximately at a constant level during the whole experiment.

Discussion. The most striking outcome of these experiments was the delay of 12 days of the time of healing in a second wound produced 6-7 days after the first one (Exp. 1). This interval is judged in comparison with the time of healing of the first wound. Compared to the 2 control groups, the interval would be even greater. This, however, is due to a fact I cannot explain, namely that the healing times of the first wounds in experiments 1-4 were consistently later, by about 4 days, than those of the controls.

It seems to follow from the experiments described, that an inhibiting effect appears during a limited period of the wound healing process, which has its influence on another healing process in the same organism. This effect may indicate the existence of an inhibiting factor appearing during this period. There is normally an inhibition during the last 7 days before closure in the first wound. In Exp. 1 a further inhibition in the second wound was observed which occurred within 1-3 days after the former. This inhibition in the second wound occurred much earlier in the wound healing process than usual, making its appearance during the phase of active proliferation of the granulation-tissue.

This inhibitory influence was not present or was too weak to become clearly visible in the Exp. 2-4, where the inhibiting period of the first wound coincided with an earlier and more active stage of the proliferation of the granulation-tissue of the second wound or with the stage immediately after the production of the second wound.

Summary. 1. Experiments were carried out in which the last phase of the healing process of a primary wound was made to coincide either with the phase of active proliferation of the granulation-tissue in a second wound in the same animal or with the stage of the very beginning of this proliferation. 2. The results obtained show the appearance of an inhibiting effect during a limited period of normal wound healing. This finding may suggest the existence of an inhibiting factor.

My thanks to Dr. G. Goldhaber (Cancer Laboratories) for his valuable help in the statistical and mathematical evaluations.

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Effect of Age upon Hepatic Synthesis of Cholesterol in Rat.* (19855)

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Although *in vitro* studies have been done on the effect of age on the hepatic synthesis of cholesterol in the rat, no similar study has been done on intact rats. Recently, however, a means was found(1) by which the hepatic synthesis of cholesterol might be gauged in the living unanesthetized rat. It consisted of collection of the daily bile output of a rat and determination of its cholesterol concentration. Other studies(2-4) have confirmed the validity of this technic, in that an increase or decrease of the biliary cholesterol concentration parallels an induced respective increase or decrease in the hepatic synthesis of cholesterol as determined by tracer studies. Accordingly, groups of rats at various ages were subjected to a study in which bile was collected and analyzed for its cholesterol concentration.

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[†] This work was begun while under tenure of American Heart Association Research Fellowship.

TABLE I. Effect of Age on Bile Cholesterol Concentration.

				Bile		
					Cholesterol	
Age	No. of rats	Wt (g)	Plasma cholesterol conc. (mg/100 ml)	Daily vol (ml)	Conc. (mg/100 ml)	Daily output (mg)
6 wk	9	116 (94-138)*	64.8 (54-88)	7.1 (5.3- 8.9)	23.6 (18.3-31.9) S.E.$_m$: ±1.5 †	1.7 (1 -2.4)
8	6	181 (161-209)	43 (32-53)	11.1 (9 -14)	26.6 (21.6-36) S.E.$_m$: ±2	2.9 (1.9-3.8)
9	12	184 (160-236)	47.7 (40-71)	12 (7.5-14.5)	24.1 (17.6-30) S.E.$_m$: ±5.2	3 (1.8-5.1)
5 mo	12	310 (253-370)	64 (52-82)	14.5 (11 -21)	16.8 (13 -20) S.E.$_m$: ±.23	2.4 (1.7-3.4)
7	10	373 (315-443)	47 (38-51)	15.7 (13.5-23)	12.8 (10.5-15.5) S.E.$_m$: ±.48	2 (1.7-2.8)
11	10	406 (319-497)	46.5 (31-59)	15.3 (11 -24.5)	12.5 (9.7-16.5) S.E.$_m$: ±.7	1.9 (1.5-2.4)

* Figures in parentheses refer to range.
† S.E.$_m$ refers to stand. error of the mean.

Methods. Male rats (Long-Evans strain) from the same colony were fed a stock laboratory ration. Groups of rats were subjected to bile duct cannulation as they attained the various ages indicated in Table I. During the ensuing 24-hour interval in which food was withheld, bile was collected and analyzed for its content of cholesterol according to technics described previously(5). In each instance the plasma cholesterol concentration was determined preoperatively(6).

Results. As shown in Table I, the bile volume output was found to increase somewhat in older rats in association with increased size and weight. Thus, the average bile volume of the immature rats (7.1-11.1 cc/24 hr) was consistently less than that obtained from the mature animals (14.5-15.7 cc/24 hr). Study of the range of values of the plasma cholesterol levels obtained in this investigation, and comparison with those obtained in other experiments performed in this laboratory fails to indicate that age had any significant effect in this respect. On the other hand, the biliary cholesterol concentration at various ages showed significant differences. Much higher average and range of values of biliary cholesterol concentration were found in the younger rats. After the age of 9 weeks, these values consistently were lower than those obtained in the immature rats. Thus, the average biliary concentration of cholesterol in the immature rats varied from 23.6 to 26.6 mg per 100 cc and, in the mature animals varied from 12.5 to 16.8 mg per 100 cc. The daily output of cholesterol in the bile, on the other hand, also is a function of the bile volume. Therefore, the daily output of biliary cholesterol was lower in the youngest group of animals despite the higher concentration of cholesterol.

Discussion. It has been observed in animals that hepatic synthesis of cholesterol depends upon the age and weight of the animal (7). Thus, in studies of cholesterol synthesis conducted in liver slices, an increased rate of synthesis was found in livers obtained from younger animals(8). The results obtained in the present investigation agree with this observation. Thus an increased hepatic synthesis of cholesterol, as reflected by a considerably greater biliary cholesterol *concentration* (1), consistently was noted in those animals in the prepubertal age groups. This was found to decrease with the advent of puberty. The study of cholesterol synthesis using the technic described above, in its agreement with the results obtained from studies employing liver slices(8), lends further proof of the validity of the biliary cholesterol concentrations as an index of hepatic synthesis of cholesterol(1).

Summary. The hepatic synthesis of cholesterol has been studied in the rat at different ages, using a biliary concentration of cholesterol as an index of this factor. The results suggest that the hepatic synthesis of choles-

terol depends upon the age of the animal, with a considerably greater synthesis in the younger rat.

The authors wish to express their thanks to Barbara Gunning, and Albert Gong, for their valuable technical assistance.

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Metabolism and Urinary Excretion of Several Flavonoid Compounds.* (19856)

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There is considerable disagreement among investigators regarding metabolism and urinary excretion of flavonoid compounds. Fukuda(1) claimed that orally administered rutin appeared unchanged in the urine of rabbits within 20 hours after administration. Garino (2) made similar claims for the dog, but Field and Rekers(3) could not confirm his findings. Porter *et al.*(4) found only traces of rutin in the urine of humans given oral doses of 60 to 2250 mg daily. Clark and MacKay(5) confirmed these findings in humans and also in the rat. Furthermore, they showed that rutin had little effect on urinary total phenol and glucuronide excretion in the rat. It was also pointed out that intestinal destruction of orally administered flavonoids made it improbable that very much of the administered dose could be absorbed and excreted in the urine. Kirtley and Peck(6) reported that hesperidin methyl chalcone was not excreted in the urine in the free form after large oral doses in man. From the above evidence it appears that the oral route of administration results in almost complete destruction of the flavonoid compounds before absorption can take place. However, it is possible that if the compounds were administered by another route, information could be derived concerning the manner in which the body metabolizes and excretes such compounds.

Experimental. Male CFW strain rats weighing an average of 294 g were caged separately in Norwich type metabolism cages. Six animals were used for each compound. Twenty-four hour urine samples were collected daily for 2 days prior to and 5 days after the subcutaneous injection of 100 mg/rat of the following compounds: rutin, hesperidin, hesperidin methyl chalcone, naringin and phloroacetophenone. Chemical analyses were made for the following: free rutin by the aluminum complex method of Porter, *et al.*(4), free hesperidin and naringin by the cyanidin test (5); free hesperidin methyl chalcone by borocitrate reaction(7); free phenol by O'Leary's method(8); ethereal sulfate by the method of Treon and Crutchfield(9); and glucuronide by Dische's method(10).

Results. The results obtained in the anlyses are given in Table I, in which the excretion as degradation products and conjugates is based upon differences between pre- and post-injection values. These are average excretion figures and represent only the first 24 hours

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* This article is based on work performed under contract between the Atomic Energy Commission and the University of California at Los Angeles.

TABLE I. Urinary Excretion of Flavonoids after Subcutaneous Injection in Rats.

Compound	Chemical name	% excreted as: Unchanged	Free phenol	Ethereal sulfate	Glucuronide	Total % excreted*
Rutin	3-rhamnoglycoside of 3,5,7,3′,4′-pentahydroxyflavone	15.89	2.57	10.90	11.15	40.51
Hesperidin	7-rhamnoglycoside of 4′-methoxy,3,5,7,3′,4′-pentahydroxyflavone	ND	4.85	11.37	9.82	26.04
Hesperidin methyl chalcone	7-rhamnoglycoside of the methyl chalcone of 4′-methoxy,3,5,7,3′,4′-pentahydroxyflavone	ND	0	3.95	4.29	8.24
Naringin	5-rhamnoglycoside of 5,7,4′-trihydroxyflavone	ND	2.82	2.87	5.12	10.81
Phloroacetophenone	2,4,6-trihydroxy acetophenone	X	22.91	33.24	22.40	78.55

* This percentage refers only to the four components measured.
ND—None detected with tests used.
X—Unchanged and free phenol were equivalent with the chemical methods used.

because no further excretion could be detected on the following 4 days. Average pre-injection control values in mg/ml of urine were: free phenol, 0.9; ethereal sulfate, 0.66 and glucuronide, 0.55. On the second post-injection day the excretion values for sulfate and glucuronide were 0.6 mg/ml and 0.3 mg/ml of urine respectively, indicating that the animals may have reached their maximum capacity for conjugation and excretion in the first 24 hours. Determinations made on the last 3 post-injection days were essentially the same as the pre-injection control values. Rutin was the only compound detected in the urine by the method of Porter, *et al.*(4). The more sensitive cyanidin reaction gave negative results with hesperidin and naringin indicating that these compounds were not excreted unchanged in the urine. Similar results were obtained with the boro-citrate reaction and hesperidin methyl chalcone. However, it is possible that if more sensitive and more highly specific reactions were available, it might be possible to detect the unchanged compounds in the urine. On the other hand, the urine from animals given rutin was highly colored, whereas that obtained from the animals receiving the other compounds was a normal straw color. None of the injected compounds could be detected when the site of injection and the surrounding tissue was excised and extracted 24 hours after administration, showing that the compounds were completely absorbed after subcutaneous injection. The free phenol values for all compounds probably represent degradation products of the flavonoids because all unchanged compounds are precipitated with and tightly bound to the gelatinous precipitate formed in the first step of the reaction. This step, which is used to clarify the urine, results in a clear filtrate which contains the free and combined phenols. Such co-precipitation was observed with urine containing rutin and it was not possible to elute the rutin from the complex precipitate. On the other hand, the free phenol values for phloroacetophenone were found to correspond to the unchanged compound and thus it appears that this compound is partially degraded by the rat into compound(s) which could not be detected with the phenol methods used.

Discussion. The results herein presented indicate that after parenteral administration, flavonoid compounds can be excreted in the urine in conjugated form and that they and/or their breakdown products are conjugated with sulfate and glucuronide in the same manner as other phenolic compounds(11). It appears that the absence of a glycoside linkage as in phloroacetophenone results in a greater excretion as a free phenol and a greater conjugation as sulfate and glucuronide. The increase in glucuronide excretion would confirm the observation of Scarborough(12) that oral vitamin P (flavonoids) increases glucuronic acid excretion. The detection of free rutin in the urine of rats makes it appear that Fukuda's(1) observations on rabbits and Garino's(2) observations on dogs are correct although the routes of administration were different. Our inability to detect hesperidin methyl chalcone in the urine indicates that Kirtley and Peck(6) were correct in assuming that the compound was not excreted in the urine in a free state. Furthermore, the small amount of the compound detected in conjugated form indicates that the compound is either stored or completely destroyed. Our results are contrary to those of Clark and MacKay(5) but the methods of administration of rutin differed. They showed that most of the rutin remained unabsorbed in the intestine after oral administration, whereas we were able to show complete absorption after subcutaneous administration. Such absorption leads to either excretion or biological destruction, and our results indicate that the latter process is the most important in the handling of hesperidin, hesperidin methyl chalcone and naringin and equivalent to excretion in the handling of rutin.

Summary. A study has been made of the metabolism and urinary excretion of rutin, hesperidin, hesperidin methyl chalcone, naringin and phloroacetophenone. Whereas the latter compound is to a large extent excreted either free or combined, the flavonoids, with the exception of rutin, are probably destroyed in the body or converted into other compounds which were not detected by the methods used. Rutin is partially destroyed in the body and partially handled by the usual phenol excretion pathways of sulfate and glucuronide.

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Chronic Toxicity of Ammonia Fumes by Inhalation.* (19857)

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The frequent exposure of industrial workers to ammonia fumes for long periods of time appears to have led to the impression that if the concentration is not sufficient to cause acute damage, then no damage need be anticipated. However, the author has been unable to find in the literature any substantiating evidence from controlled experimentation. In view of the fact that Fazekas(1,2) found that the administration of 50-80 cc of 0.5% ammonium hydroxide orally to rabbits either daily or on alternate days for periods of several months caused a marked increase in size of the suprarenal glands, it would appear desirable to investigate the potential toxicity of ammonia by inhalation. Accordingly, this was done.

Experimental methods. Twelve male guinea pigs were used for experimentation and 6 for controls. All animals were fed Purina rabbit chow supplemented with greens. The con-

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trols were denied food and drink during the periods that the experimental animals were in the gas chamber. All animals were weighed weekly. Exposure to ammonia was for 6 hours per day, 5 days per week. At intervals of 6 weeks 4 experimental and 2 control animals were sacrificed, and hearts, lungs, livers, stomachs, small intestines, spleens, kidneys, and suprarenal glands were removed for microscopic examination. Anhydrous ammonia gas was led from the cylinder of liquid ammonia through a flow-meter into the gas chamber where it was discharged into the air stream from a centrifugal blower. Concentration of ammonia in the chamber atmosphere was measured 2 or 3 times daily by chemical analysis. A chamber concentration of 170 parts per million by volume was sought. By actual analyses the concentration varied between 140 and 200 p.p.m., with only occasional values falling beyond these limits. Henderson and Haggard(3) report the least concentration necessary to be detectable by odor as 53 p.p.m. and to cause immediate throat irritation as 408 p.p.m. Five or 6 members of the laboratory staff inhaled the exhaust fumes from the chamber, and all were of the opinion that no person would remain in such an atmosphere voluntarily for any appreciable period of time because of the disagreeable odor and respiratory distress. Therefore, it appears probable that such a concentration represents an approximate maximum which one might have to contend with as a potential cause of chronic intoxication.

Results. Animals sacrificed after 6 and 12 weeks of exposure showed no abnormalities which could be attributed to ammonia. There were the minor lesions of one sort or another which are common in most laboratory animals and of no special significance. However, those animals which had been exposed to ammonia for 18 weeks exhibited changes which were not observed in the controls nor in others sacrificed after the shorter periods of exposure. There was considerable congestion of spleens, livers and kidneys, and early degenerative changes in the suprarenal gland. The spleens consistently contained larger than normal quantities of hemosiderin, which is taken to indicate increased blood destruction. The upper tubules of the kidneys and in 2 of the 4 animals the lower tubules as well showed fairly marked cloudy swelling with precipitated albumen in the lumens and some casts. In 2 of the animals there was a brownish discoloration of the blood in the vessels of the medulla between the connecting tubules suggestive of the formation of hematin. The changes in the suprarenal glands were not marked, and consisted of swelling of the cells with degeneration of the cytoplasm of the cells of the mid and inner zones. This degeneration was of the nature of a loss of normal granular structure of the cytoplasm giving to it a more nearly homogeneous appearance. The suprarenal glands were not of abnormally large size. Histological studies were made by Dr. G. Z. Williams, Prof. of Pathology, in this institution.

Summary. Exposure of male guinea pigs to an atmospheric concentration of 170 p.p.m. of ammonia for periods of 6 hours per day, 5 days per week for as long as 12 weeks gave no significant evidence of chronic intoxication. However, such exposure for 18 weeks resulted in relatively mild though definite changes in spleens, kidneys, suprarenal glands, and livers, with severity of the changes being most prominent in spleens and least in livers. Hearts, lungs, stomachs, and small intestines showed no consistent changes suggestive of chronic intoxication.

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Use of Ethylene Oxide for Preparation of Stable Non-Infective Influenza and Mumps Soluble Antigens. (19858)

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Ethylene oxide assumed importance as a biological agent in 1928 when it was described as a powerful insecticide against a large number of insect pests(1). Its use for this purpose spread rapidly. Later its activity against molds was reported(2). It soon became widely used for the sterilization of spices and other materials in a vacuum chamber process(3,4). Schrader and Bossert(5) first described the use of ethylene oxide as an antibacterial agent. This work was extended with the reports of its use to sterilize soil(6) and various culture media(7). In all the above reports, however, ethylene oxide was employed in the gaseous form. The first use of liquid ethylene oxide for the sterilization of media and other fluids was described by Wilson and Bruno(8). Later it was reported that 1% by volume of liquid ethylene oxide inactivated numerous viruses(9).

A method for the preparation of stable non-infective soluble influenza antigen involving the use of formaldehyde followed by its neutralization prior to lyophilization has been described previously(10). If other agents such as liquid ethylene oxide could be used for this purpose the procedure would be simplified. Consequently, an investigation was made to seek conditions of treatment with ethylene oxide by which stable dry non-infective soluble antigens could be prepared.

Materials and methods. The soluble antigen was prepared from the chorioallantoic membranes of 13-day-old chick embryos which had been infected with the PR8 strain of influenza A virus by the allantoic route 2 days previously. To the pooled membranes was added 1.5 ml of saline per membrane and the mixture was ground for 2 minutes in a Waring blendor. After a preliminary centrifugation for 10 minutes at 2000 r.p.m., the supernatant suspension was stored in the cold room at 4°C for 4 days. Any precipitate formed was then removed by another centrifugation at low speed, after which the supernatant suspension was centrifuged for one hour at 15,000 r.p.m. The supernatant liquid was removed and used as the soluble antigen. Infectivity tests on untreated controls and samples treated with ethylene oxide were conducted by direct inoculation of undiluted and 10^{-2} diluted suspensions into the allantoic cavity of 10-11-day-old chick embryos, with a subsequent incubation for 72 hours. Samples which after 2 successive passages yielded allantoic fluids free of specific hemagglutinins were considered to be non-infective. The following experiment was conducted to determine the optimal experimental conditions of ethylene oxide concentration, temperature, and time of treatment required to yield a soluble antigen which could be lyophilized and stored, and which when redissolved would be non-infective and would have undergone a minimal loss of antigenicity. A 50% solution of ethylene oxide (Eastman Kodak) was freshly prepared in distilled water at 4°C. This solution was added to 3 samples of a common pool of PR8 soluble antigen, precooled to 4°C, to give final concentrations of 1%, 3%, and 5% by volume of ethylene oxide. The ethylene oxide was added in the form of a 50% aqueous solution to facilitate accurate transfer with precooled glassware. Aliquots of each antigen sample were then placed in cotton-stoppered test tubes in water baths at 37°C, 25°C, and in the cold room at 4°C. Samples were removed after various periods of time and were lyophilized immediately in an Edwards centrifugal freeze-dryer, thereby removing all ethylene oxide. The dried antigens were reconstituted to their original volume with distilled water and tested for specific antigenicity in the complement-fixation test.

Results. It was found from these equipments that: 1) the samples of antigen stored at 4°C up to 24 hours showed no decrease in titer, 2) with the samples stored at 25°C, the

TABLE I. Effect of Various Conditions of Ethylene Oxide Treatment on PR8 Soluble Antigen.

Ethylene oxide vol, %	Temp., °C	Time of treatment (hr)	No. antigens tested	No decrease in titer	50% decrease in titer	>50% decrease in titer	No. of antigens containing live virus after treatment
5	4	16	9	7	1	1	1
5	4	24	22	21	1	0	0
5	4	48	13	12	1	0	0
5	25	2	5	3	1	1	1
3	25	2	17	13	4	0	3
3	25	24	14	8	5	1	0
3	37	2	20	10	10	0	1
10	4	24	8	8	0	0	0

antigen containing 5% ethylene oxide showed a progressive decrease in titer with time but the antigens containing 3% and 1% ethylene oxide underwent no significant decrease, 3) with the samples at 37°C, a decrease in titer was observed after 2 hours with the 3 concentrations of ethylene oxide used and the antigenicity was almost completely destroyed after 24 hours.

This type of experiment was then repeated using several different lots of PR8 soluble antigen to which there was deliberately added viable PR8 virus which resulted in suspensions with hemagglutinin titers of 1280 units per ml or greater. After lyophilization, the ID_{50} of 4 different untreated antigens were found to be approximately $10^{-8.6}$ to $10^{-9.8}$ per ml by allantoic titration. Only ethylene oxide concentrations and temperatures which had been found previously not to cause a significant loss of antigenicity were used in this experiment. The samples stored at 4°C were loaded into ampoules at room temperature, the total time at room temperature being 30 minutes. This step was introduced for the convenience of handling large lots. In an additional experiment, a concentration of 10% by volume of liquid ethylene oxide was employed, obtained by direct addition of the oxide in the undiluted form to the antigen. After lyophilization, these antigens were reconstituted and tested for antigenicity and infectivity as described above. The results are summarized in Table I.

From Table I it can be seen that several combinations of experimental conditions come close to fulfilling the desired criteria for a method of rendering these antigens non-infective with little or no loss of antigenicity. Treatment with 3% ethylene oxide at 37°C for 2 hours destroyed the infectivity in all cases except one, but half of the treated antigens showed a 50% decrease in complement-fixation titer. A concentration of 3% ethylene oxide at 25°C for 24 hours was found to be effective for destroying the infectivity but there was a decrease of antigenicity in some antigens. However, it is evident that the best results in preparing non-infective antigens can be obtained by the addition of 5% by volume of liquid ethylene oxide and treatment at 4°C for 24 or 48 hours, followed by a 30-minute period at room temperature to allow for ampoule filling and then immediate lyophilization. Since one sample was found to be still infective after 16 hours, it is considered that the 48-hour period of treatment represents the method of choice as it provides a wider margin of safety than the 24-hour treatment.

These experiments were repeated on 10 different lots of mumps soluble antigen to which were added viable mumps virus to give hemagglutinin titers of 640 to 1280 units per ml. Here it was found that only the treatment with 5% ethylene oxide for 24 hours at 4°C was successful in destroying the infectivity in all cases while the antigenicity was not decreased. The period of treatment could also be extended to 48 hours with no loss of titer except in one case where the antigen had been stored for 11 months in a deep-freeze at —20°C prior to its present use.

Discussion. In the biological field in the past, ethylene oxide has been employed primarily as a fungicide, insecticide, and germicide, that is, as a disinfectant. In the present investigation conditions of ethylene oxide treatment have been sought which would permit the destruction of viral infectivity while at the same time preserving the antigenicity. No reaction conditions below a 24-48-hour period of treatment have been found which would achieve this double objective. However, treatment of the soluble antigens of influenza and mumps with 5% by volume of liquid ethylene oxide at 4°C for 48 hours followed by a 30-minute period at room temperature to allow for ampoule filling and then immediate lyophilization has been found to yield antigens which when reconstituted to their original volume with distilled water are non-infective and have undergone no significant decrease in complement-fixation titer in the process.

At the time of writing, antigen samples treated as described above have been stored in the cold room at 4°C for 3 months without significant loss of titer.

Summary. An investigation has been conducted of the use of liquid ethylene oxide for the preparation of stable non-infective soluble antigens of influenza and mumps. It has been found that treatment of these antigens with 5% by volume of liquid ethylene oxide at 4°C for 48 hours, then for 30 minutes at room temperature, followed by immediate lyophilization, is a satisfactory method to accomplish this purpose.

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Influence of Nitrogen Mustard upon Reactions to Bacterial Endotoxins: Shwartzman Phenomenon and Fever.* (19859)

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Becker(1) found that the Shwartzman phenomenon could not be elicited in rabbits a few days after injection of nitrogen mustard (hereafter referred to as HN_2) or benzol or after total body x-ray irradiation. This effect has been confirmed by Schlang(2) and by Stetson and Good(3). Although Becker postulated that the effect of HN_2, benzol and x-ray was due to the production of an "anergic" state in cells of the vascular endothelium, Stetson pointed out that all 3 of these forms of treatment are followed in a few days by a profound neutropenia. His experiments showed that the period of Shwartzman non-reactivity after HN_2 or benzol coincides with the time of maximum depression of circulating granulocytes. Furthermore, prevention of leukopenia by aortic compression in HN_2-treated animals or by administration of sulfapyridine in benzol-treated rabbits eliminated the period of Shwartzman non-reactivity. Stetson concluded that the presence of an adequate number of circulating granulocytes is necessary for the elicitation of the Shwartzman reaction and that the inhibitory effect of HN_2 and benzol is due to suppression of these cells. Weis-

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berger(4) reported that rabbits given l-cysteine showed less depression of circulating granulocytes after HN_2 than untreated animals.

The present report describes the effect of pretreatment with l-cysteine upon HN_2 inhibition of the Shwartzman phenomenon. Injection of colloidal materials such as thorotrast abolishes natural resistance in rabbits to the Shwartzman reaction as well as resistance acquired in the course of repeated elicitation of the reaction(5,6). The mechanism of this effect is not entirely clear although an effect on the reticulo-endothelial system may play a part. Its influence on the non-reactivity produced by HN_2 was investigated. Bacterial toxins capable of eliciting the Shwartzman phenomenon produce transient leukopenia and fever when injected intravenously in rabbits (7). It was therefore thought that observation of the effect of HN_2 induced leukopenia upon the febrile response to bacterial pyrogens might yield some information about the importance of circulating leukocytes in the febrile reaction.

Materials and methods. White male rabbits of mixed breed weighing 2-3 kg were used. Hair was removed from the abdomen with electric clippers and successive intradermal injections were made in alternate sides of the abdomen beginning in the right upper quadrant. Material used to elicit the Shwartzman reaction was a filtrate of agar washings from a culture of *Serratia marcescens*(8). The intradermal preparatory dose was 0.5 ml of 1/10 dilution and the intravenous dose, given 24 hours later, was 1.0 ml of 1/25 dilution. With this dosage, approximately 90% of normal animals tested showed typical Shwartzman lesions. In some of the experiments on fever, a vaccine containing 1.0 billion typhoid bacilli per ml was used and in others P-25 polysaccharide from *S. marcescens* (obtained from Dr. M. J. Shear) was employed. HN_2 (mechlorethamine hydrochloride, Merck) was freshly prepared in a concentration of 1.0 mg/ml in distilled water. This solution was injected rapidly into a marginal ear vein in a dose of 5.0 mg. l-Cysteine hydrochloride was prepared in 20% solution, neutralized to pH 7.0 by addition of sodium hydroxide. This was injected in a dose of 650 mg/kg intraperitoneally in rabbits, 20 minutes before administration of HN_2. Leukocyte counts were performed a few minutes before injection of HN_2 or cysteine, and daily thereafter. The maximum leukopenic effect was usually apparent from the third to fifth day, following the pattern which has been described frequently (3,4,9). On days when P-25, *S. marcescens* filtrate, or typhoid vaccine was to be given, counts were done before injection. Skin lesions were classified as positive only if there appeared an area of confluent hemorrhage at the prepared site. In experiments on fever, animals were placed in wooden stalls, secured with loose-fitting collars and rectal temperatures were taken every 30 minutes for 6 hours by means of ordinary clinical thermometers. In order to facilitate comparison of temperature responses before and after HN_2, fever curves were charted on 3/16 inch graph paper and, using the temperature at the time of injection as a base line, the area beneath each curve was measured with a Keuffel and Esser compensating planimeter (No. F4236). The vernier reading of the planimeter was taken as the "fever index", an expression of the height and duration of fever. Thorotrast, a 25% suspension of thorium dioxide (Heyden Co.), was injected slowly intravenously in a dose of 9.0 ml 8 hours after the intradermal preparatory injection for the Shwartzman reaction and 16 hours before the intravenous provocative injection.

Certain difficulties should be mentioned. Of 78 rabbits given HN_2 alone, 49 died before experiments could be completed. Of these, 20 died after HN_2 injection only and 29 after one or more injections of one of the bacterial materials employed in the study. Of 67 rabbits given HN_2 after pretreatment with cysteine, 43 died before completion of experiments, 25 after cysteine and HN_2 only and 18 after one or more injections of bacterial toxin. Despite the neutralization of cysteine solutions to pH 7.0, injection of this material into the peritoneal cavity of rabbits was attended by evidence of considerable irritation, at times accompanied by mild circulatory collapse within a few minutes after injection. It was found in later experiments that these symptoms could be ameliorated by making the intraperitoneal injection while the animals were under light nembutal anesthesia.

Experimental. Effect of l-cysteine upon inhibition of the Shwartzman phenomenon by HN_2. Forty rabbits which had demonstrated positive Shwartzman reactions when tested with *S. marcescens* filtrate were divided into 2 equal groups designated A and B. Animals in group A were given only HN_2 intravenously and those in group B, after pretreatment with l-cysteine, were also given HN_2. Beginning on the first day after injection of mustard, the 2 animals showing the lowest leukocyte counts in each group were tested for capacity to exhibit the Shwartzman phenomenon. After the skin reaction was noted, animals were then allowed to rest until the 9th day after HN_2 injection when intradermal filtrate was again given, followed by intravenous provocative dose on the 10th day. This procedure was continued, 2 animals of those remaining in each group being tested daily and all animals being retested on the 9th and 10th days. Because of deaths before completion of the 10-day experimental period, it was necessary to carry out this procedure in 2 additional groups of animals. Animals given HN_2 alone showed marked leukopenia from 2 to 6 days later and during this period the Shwartzman reaction was negative. All animals showed typical Shwartzman reactions when retested on the 9th and 10th days. In contrast, the leukopenic effect of HN_2 was much less in the cysteine-treated animals of group B and with one exception, an animal whose count fell to 400 on the second day, all animals given cysteine responded to injection of *S. marcescens* filtrate with hemorrhagic necrosis of the Shwartzman type. Table I shows the results obtained in group B.

TABLE I. Effectiveness of Premedication with l-Cysteine in Preventing Inhibition of Shwartzman Reactivity by HN_2. Note that the only negative reaction was in an animal with leukopenia.

Days after HN_2	WBC on day of: Skin inj.	WBC on day of: I.V. inj.	Reaction
1, 2	4250	5550	+
	2050	5200	
2, 3	400	1100	—
	1350	8000	+
3, 4	1300	2050	
	2400	2750	
	1600	5600	
4, 5	3100	10450	
	2050	24000	
5, 6	4600	6450	
	5600	7950	
6, 7	5000	14960	
	5600	3500	
7, 8	6700	17880	
	6250	5100	
9,10	3100	4150	
	5050	9050	
	1950	2200	
	15850	15440	
	4300	11750	
	6450	7000	
	8600	3350	
	12840	11120	
	9950	7500	
	10400	1200	
	7300	11600	
	9200	9500	
	4850	9700	
	12000	7100	
	8700	13450	

Failure of thorotrast injection to alter HN_2 inhibition of the Shwartzman reaction. Eight animals that had demonstrated positive Shwartzman reactions with *S. marcescens* filtrate were given 5.0 mg HN_2 intravenously and followed with daily leukocyte counts. On the 4th day, at which time 6 animals showed peripheral counts of less than 1000, all received bacterial filtrate intradermally and, 8 hours later, each one was given thorotrast. Twenty-four hours after skin preparation, leukocyte counts were repeated and *S. marcescens* filtrate was given intravenously. None showed a positive Shwartzman reaction at the prepared skin site, indicating that thorotrast does not overcome the inhibitory action of HN_2. Four of the 8 animals were retested with *S. marcescens* filtrate on the 9th and 10th days after HN_2 injection, at which time their leukocyte counts had risen to normal figures. All reacted with typical skin hemorrhage.

Effect of HN_2 leukopenia upon pyrogen-induced fever. The febrile response of rabbits after injection of 0.05 ml of typhoid vaccine or 0.005 mg of P-25 polysaccharide was measured and, after a rest period of 1 week, animals were given 5 mg of HN_2 intravenously. Daily leukocyte counts were performed in all animals and, when the total peripheral count fell below 300/mm^3, the injection of vaccine or P-25 was repeated and temperatures recorded. Leukopenic animals responded

TABLE II. Fever Indices after Injection of .05 ml Typhoid Vaccine in Rabbits before and after HN_2. Peripheral leukocytes were reduced below 300/mm^3 by HN_2.

	Before HN_2	After HN_2
	168	156
	192	176
	243	216
	186	220
	212	197
	161	191
	144	163
	212	176
	149	170
	198	162
Avg	186.5	182.7

to pyrogen injection with brisk fevers which could not be distinguished from those before HN_2 injection. Table II compares the febrile responses of 10 rabbits to typhoid vaccine before and after HN_2 treatment. Similar results were obtained in 6 animals given P-25 polysaccharide. These findings indicate that the production of fever by bacterial materials is not dependent upon the presence of a normal number of leukocytes in the peripheral circulation.

Discussion and summary. The finding of Stetson and Good that the period of inhibition of the Shwartzman phenomenon which follows injection of HN_2 in the rabbit coincides with the time of maximum depression of circulating leukocytes was confirmed. In addition, the prevention of severe leukopenia by cysteine resulted in retention of Shwartzman activity, further evidence for the role of the leukocyte in the Shwartzman reaction. Although injection of colloidal materials abolishes natural or acquired resistance to the Shwartzman phenomenon, thorotrast did not influence the Shwartzman non-reactivity produced by HN_2. The failure of HN_2-induced leukopenia to influence the febrile response of rabbits to pyrogenic bacterial materials indicates that pyrogen fever is not dependent upon the presence of normal numbers of circulating leukocytes.

Mrs. Frances Brown and Mrs. Anne McManus gave technical assistance in this work.

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Beneficial Effects of Vitamin B_{12} and Aureomycin in Rats Given Large Doses of Cortisone.*† (19860)

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On corn-soybean meal or corn-powdered milk diets, vit. B_{12} or antibiotics have been shown to be partially or completely effective

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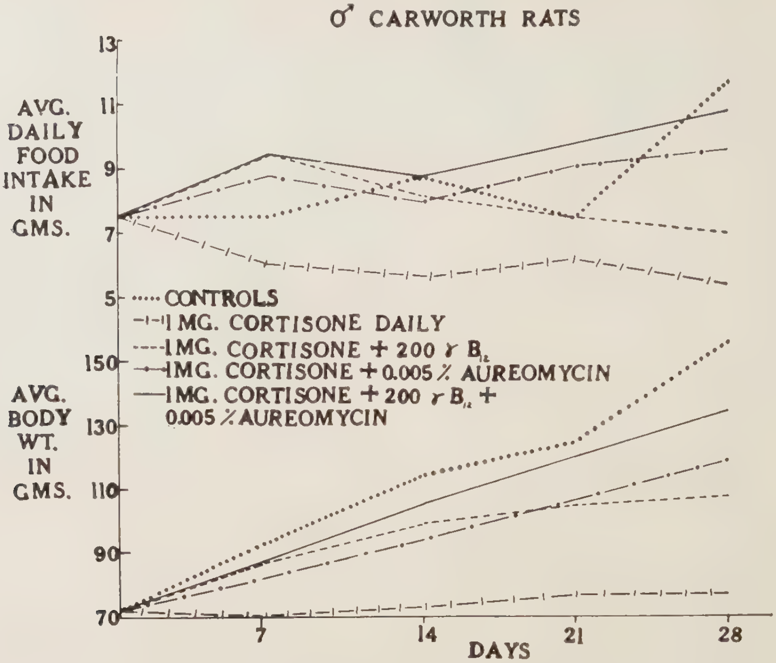

FIG. 1. Graph showing avg body wt and food intake of each group. Note complete inhibition of growth and reduction in appetite of the group which received cortisone alone, and improvement with vit. B_{12} and aureomycin.

in counteracting growth inhibition induced by administering large doses of iodinated casein, thiouracil, diethylstilbestrol or cortisone(1,2). These effects of the vitamin or antibiotics were usually accompanied by an increase in food intake and by greater efficiency in converting food into body-weight gains.

On a purified diet containing casein as the dietary protein and sucrose as the carbohydrate, Ershoff(3) reported that whole liver but none of the known B vitamins largely counteracted inhibition of body growth and alopecia in young rats fed massive doses of cortisone. Ershoff(3) suggested the presence of a separate protective factor in the liver different from all known B vitamins. This paper reports the results of an experiment in rats fed a purified diet containing casein as the dietary protein and glucose as the carbohydrate. On this diet it was found that vit. B_{12} and/or aureomycin were effective in largely counteracting inhibition of body, hair and thymus growth induced by injecting large doses of cortisone.

Methods. Fifty young male Carworth rats were divided into 5 uniform groups of 10 each, and placed on the following purified diet: alcohol washed casein,[‖] 25 g; glucose (Cerelose),[¶] 62 g; corn oil (Mazola), 5 g; cod liver oil,[**] 5 g; salt mixture,[††] 4 g. To each kilo of the above, the following vitamins were added: choline 1.0 g; thiamin, 2.0 mg; riboflavin, 5.0 mg; pyridoxine, 2.0 mg; niacin, 10.0 mg; Ca pantothenate, 28 mg; 2-Me-1,4 Naphthoquinone, 0.4 mg. After being on the above diet for a preliminary period of 2 weeks, each of the 5 groups was treated as follows for 28 days: 1, controls; 2, 1 mg cortisone acetate[‡‡] daily by subcutaneous injection; 3, 1 mg cortisone acetate daily and 200 μg vit. B_{12} per kilo of

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[‖] "Vitamin Test" casein, Nutritional Biochemicals Corp., Cleveland, O.

[¶] Corn Products Refining Co., Chicago, Ill.

[**] Contains 2000 USP units of vit. A and 250 units vit. D/g.

[††] Salt mixture No. 2, Nutritional Biochemicals Corp., Cleveland, O.

[‡‡] The B vitamins and cortisone acetate (Cortone) furnished through the kindnesss of Dr. E. Alpert of Merck and Co., Rahway, N. J.

diet; 4, 1 mg cortisone acetate daily and 0.005% aureomycin HCl[§§] in the diet; 5, 1 mg cortisone acetate daily, 200 μg vit. B_{12} per kilo of diet and 0.005% aureomycin HCl. Individual body weights and food consumption were measured once weekly. Eosinophil counts were made from tail blood on the last day of the experiment. After killing the rats with ether, the adrenals and thymus glands were removed and weighed, and the latter were fixed for histological examination. All rats were maintained in metal cages with raised screen bottoms in an air-conditioned room at a temp. of 75 ± 2°F.

Results. Average body growth and food intake are shown in Fig. 1. All rats weighed approximately 71 g at the beginning of the experiment. The average final body weight and total food consumption per rat were as follows for each group: 1, controls, 155.4 ± 5.6 and 240.8 g; 2, cortisone acetate, 77.0 ± 4.2 and 162.4 g; 3, cortisone acetate and vit. B_{12} 107.5 ± 4.4 and 204 g; 4, cortisone acetate and aureomycin HCl, 117.2 ± 4.2 and 243.6 g; 5, cortisone acetate and vit. B_{12} and aureomycin HCl, 135.3 ± 6.8 and 251.2 g. It can thus be seen that cortisone completely suppressed body growth and reduced food intake, while supplementation of the diet with vit. B_{12} and/or aureomycin significantly counteracted these effects of the hormone.

Fig. 2 shows the typical appearance of 5 representative rats from each of the 4 cortisone-treated groups. It can be seen that the rats given cortisone alone are not only stunted in size but have developed a severe alopecia which was particularly marked over the head, neck and back areas. Either vit. B_{12} or aureomycin markedly reduced the alopecia, while the combination of the two appeared to completely eliminate it.

The effects of cortisone alone or in combination with vit. B_{12} or the antibiotic on the thymus gland are shown in Fig. 3. In the group treated with cortisone alone, there was marked atrophy of the thymus, disappearance of the cortical area and a great reduction in thymocytes. In the cortisone treated groups whose diets were supplemented with the vitamin and/or antibiotic, the thymus glands were more normal in size and appearance. Distinct cortical and medullary areas were present and the number of thymocytes was increased. The average thymus weight per 100 g of rat for each of the 5 groups was as follows: 1, 216.3 mg; 2, 52.0 mg; 3, 111.6 mg; 4, 115.6 mg; 5, 142.8 mg.

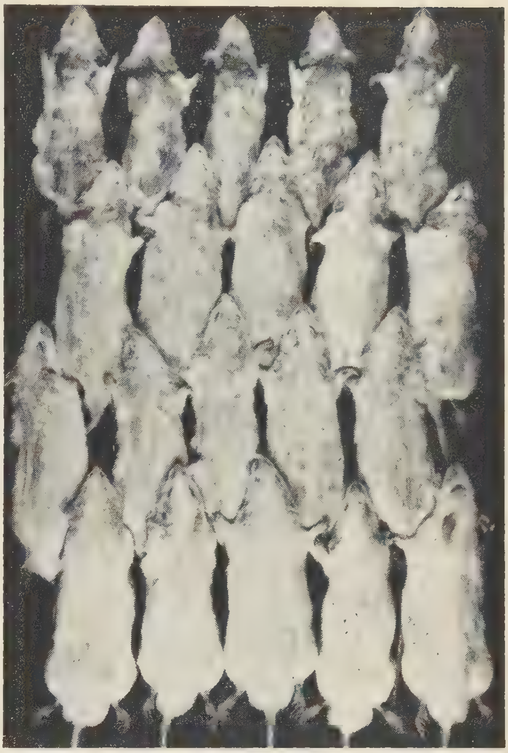

FIG. 2. Appearance of representative rats in the exp. From top to bottom, each group was treated as follows: Cortisone only; cortisone and vit. B_{12}; cortisone and aureomycin; cortisone, vit. B_{12} and aureomycin. Note the significant improvement in hair and body size of the rats given vit. B_{12} and/or aureomycin.

Eosinophil counts were reduced from an average of 330 per cu ml in the control rats to 28 in the rats given cortisone alone. Neither vit. B_{12} nor aureomycin significantly altered this action of cortisone on the eosinophils. The average weight of the adrenals of the control rats was 32.9 mg as compared to 18.8-23.4 mg in the rats given cortisone with or without vit. B_{12} and aureomycin.

Discussion. The results of this experiment, in which a purified casein-glucose diet was

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[§§] Kindly supplied by Dr. T. H. Jukes of Lederle Labs. Division, Pearl River, N. Y.

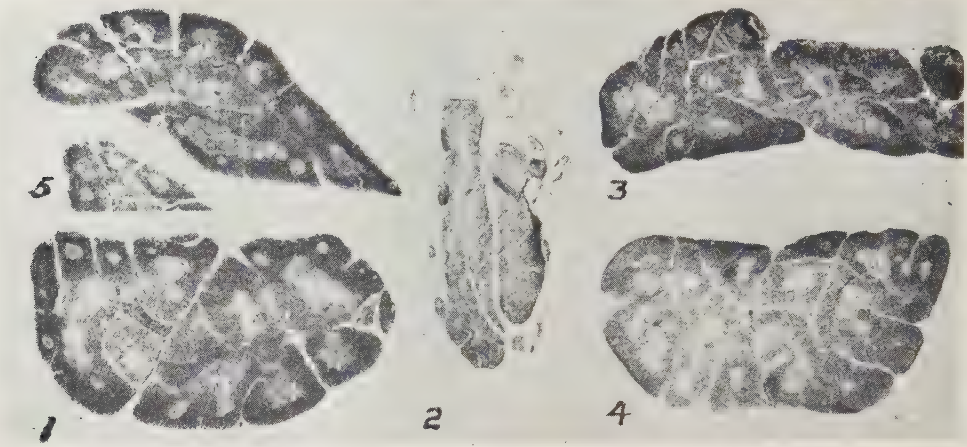

FIG. 3. Histological sections of representative thymus glands from each of the 5 groups. Treatment was as follows: 1, control; 2, cortisone only; 3, cortisone and vit. B_{12}; 4, cortisone and aureomycin; 5, cortisone, vit. B_{12} and aureomycin.

fed, are in general agreement with the data previously reported in rats fed a corn-soybean meal diet(1,2). In both experiments, vit. B_{12} and/or aureomycin largely protected young male rats against the inhibitory effects of large doses of cortisone on body, hair and thymus growth. It is of interest that on the corn-soybean meal diet, the dose of cortisone used (0.5 mg daily) did not induce alopecia, although it decreased the rate of hair regrowth on the backs of rats from which it had initially been removed. Even 1 mg of cortisone daily did not induce alopecia in rats fed this diet, although it completely inhibited body growth (unpublished observations). On both diets, the combination of vit. B_{12} and aureomycin was more effective than either alone in overcoming cortisone "toxicity."

Ershoff's(3) inability to counteract to any degree the inhibitory effects of huge doses of cortisone with combinations of the known B vitamins, including vit. B_{12}, is at variance with the results reported here.[‖‖] However, the diets and strain of rats employed in the two laboratories were different. Previously, Ershoff(4) had reported that when his same casein-sucrose diet was used, thyrotoxicosis in young rats could be overcome by whole liver but not by Vit. B_{12}. Other workers, however, could at least partially counteract thyrotoxicosis with vit. B_{12} on casein-sucrose (5,6), casein-glucose(7), soybean meal(6,8,9) or corn-whole milk(10) diets. In commenting on the differences between their results and those of Ershoff(4), Lewis *et al.*(6) suggested that altering the carbohydrate in the diet may change the intestinal synthesis of other factors. Furthermore, the favorable results obtained with whole liver might possibly be due to its glycogen content, since activity was reported only when liver was fed at a 10% level. It would be of interest to determine whether liver has any advantage over vit. B_{12} and aureomycin in counteracting large doses of cortisone in rats fed the casein-glucose diet of the present experiment.[¶¶]

It is believed that the large doses of cortisone given the rats in this experiment increased their requirements for vit. B_{12} and other factors. A similar opinion has been expressed regarding the effects of marked changes in levels of other hormones on nutri-

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[‖‖] Ershoff's own data indicate that supplementation with the known B vitamins enabled his female rats to at least partially overcome inhibition of body growth induced by feeding 100 mg of cortisone acetate per kg of diet.

[¶¶] In a similar experiment just completed, 10% whole liver powder was found to be more effective than either vit. B_{12} or aureomycin in counteracting cortisone in rats. Best results were obtained by combining all 3 factors in the diet. The question as to whether liver contains a factor different from all known B vitamins or whether its carbohydrate, fat or proteins account for its effectiveness remains unanswered.

tional requirements(2). Wahlstrom and Johnson(11) had previously observed that large doses of cortisone injected into baby pigs on a vit. B_{12}-deficient diet increased the urinary excretion of vit. B_{12} and reduced body growth and survival rate. Since it is well established that cortisone can increase the loss of body protein, as indicated in this experiment by retardation of body, hair and thymus growth, the favorable effects of vit. B_{12} can perhaps be attributed to an essential role in protein(12,13) and nucleic acid(14, 15) synthesis. Aureomycin and other antibiotics have been shown to exert a sparing action on several B vitamins, including vit. B_{12}(16-18). It is of interest that increased food intake(19), testosterone(19,20) and growth hormone(21,22) have all been observed to protect the body against the catabolic actions of ACTH and cortisone in a manner similar to that reported here for vit. B_{12} and aureomycin. It seems possible that the factor which all of these have in common is their ability to enhance protein retention within the body.

Summary. 1. Fifty young male albino rats were uniformly divided into 5 equal groups and placed on a purified casein-glucose diet. After a preliminary period of 2 weeks, all except a control group were injected for 28 days with 1 mg of cortisone acetate daily with or without vit. B_{12} and/or aureomycin. 2. Cortisone acetate alone induced complete inhibition of body growth, severe alopecia and marked atrophy of the thymus gland. Vit. B_{12} (200 μg/kilo diet) or aureomycin (0.005%) largely counteracted these inhibitory effects of cortisone acetate, and the combination of the two was more effective than either alone(3). It was concluded that large doses of cortisone acetate given to young rats on a casein-glucose diet, increased their requirements for vit. B_{12} and other factors. These data corroborate results previously obtained in a similar experiment on rats fed a corn-soybean meal diet.

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Effects on Growth of Chicks and Poults of Feeding Yeast Isolated from Streptomycin-Fed Poults.[*] (19861)

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It has been shown repeatedly that the addition of antibiotics to rations which are believed to contain adequate amounts of all the known nutrients, results in a distinct growth response by chicks or turkey poults,(1-4). Concomitant with this growth increase, when streptomycin was fed to poults, a 5- to 10-fold increase in the yeast population of the intestinal contents was noted, as compared with the control birds which did not receive the antibiotic in their diet. Although cultivatable yeasts represent a very small component of the intestinal microflora, a closer examination of their possible role in the antibiotic growth response was prompted because commercial yeast cultures have been reported to produce a small growth response when added to normal diets(5). The following investigations were undertaken to determine whether the feeding of live cultures of yeast, originally isolated from streptomycin-fed poults, could increase the growth rate in a manner similar to that of the antibiotics themselves.

Preparation of the cultures. Yeasts were isolated from the intestinal contents and feces of poults which had been fed a ration containing 50 mg of streptomycin per kilo of diet. In one procedure, samples of intestinal contents were obtained, immediately after sacrificing, from the region just anterior to the ileocecal junction. In another, changes in the fecal flora were being studied by periodic culture from hatching until 5 to 6 weeks. Droppings from these poults were transferred to sterile containers immediately after defecation. Both types of samples were cultured in the same manner. Quantitative platings were made using 2 specialized media: wort agar; and acidified potato-dextrose agar (pH 3.5). Microscopic examination of preparations from individual colonies showed that many yeasts were present. A representative collection of these yeasts was assembled from the quantitative plates, and the cultures were purified by restreaking several times from aqueous suspensions. Most of these cultures were, superficially at least, alike in microscopic morphology and colony appearance and, for economy in futher work, a single representative culture was studied most intensively. This organism designated as E-16, was grown in mass culture for poultry feeding trials, and subjected to the usual microbiological determinative tests. Occasional use was made of an unidentified yeast-like fungus, designated G-1-B, which was isolated from a commercial yeast supplement. Unless otherwise qualified, however, the results detailed here are based on studies with yeast strain E-16. *Mass cultivation.* For the poultry feeding trials, the yeasts were grown in a yeast-peptone broth[†] distributed in 1 liter volumes in Fernbach flasks. They were incubated at about 30°C on a rotary shaking machine for several days. When the population had attained a maximum cell volume, the yeast was harvested by means of a Sharples supercentrifuge. The moist cake was stored at 4°C or if dried material was desired, lyophilized in a freeze-dry apparatus (8). Contrary to expectations, the viability of the yeast was not reduced appreciably by lyophilization. Approximately 20 g (wet weight) of cells per liter of medium were produced in this way. *Determinative Tests.* The typical culture (E-16), was subjected to the usual taxonomic examination, with results as follows: Moist creamy growth on potato-

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[*] Streptomycin and procaine penicillin were kindly supplied by Merck and Co., Rahway, N. J., through the courtesy of Dr. D. F. Green and crystalline aureomycin and APF supplement by Lederle Laboratories Div., American Cyanamid Co., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

[†] Formula for yeast-peptone broth:

Yeast extr.	5 g	K_2HPO_4	2
Trypticase	5	$CaCl_2$	.2
$(NH_4)_2HPO_4$	1	Glucose (cerelose)	20
$MgSO_4 \cdot 7H_2O$	.5	Water *q.s.*	1 liter

dextrose agar; colonies entire and convex. Dull, pasty growth on yeast-dextrose-$CaCO_3$ agar; somewhat spreading colony. Mycelium present in subsurface growth after several days at room temperature. Many budding cells along the hyphae; some pseudomycelium and true mycelium. Surface growth on corn meal agar consists of small, pasty colonies with a subsurface mycelium, no chlamydospores observed after five days at 25°C. Surface growth on blood agar (37°C) shows small, entire colonies. A mycelial fringe is noted after 6 days with budding cells on the mycelium. Acid and gas are produced from glucose; no acid or gas from lactose or raffinose. No ascospores observed on carrot or potato slices, on plaster of paris, or in tomato juice with a paper wick. Subcutaneous injection of a dense suspension of yeast cells into guinea pigs produced no lesions. On the basis of these tests and observations, yeast strain E-16 is tentatively identified[‡] as *Candida tropicalis.*

Procedure for growth tests. Samples of yeasts were tested for growth-promoting activity in 4 different series of experiments, using both chicks and poults. The chicks used were S.C. White Leghorn cockerels unless noted otherwise; the poults were of the Bronze variety not segregated by sexes. The birds were housed in electrically heated batteries with raised wire floors. Some of the experiments were started when the birds were one day of age, while in others the control ration was first fed for a week or 10 days. In Series 1, the yeast preparations were mixed in the feed at the start of the experiment with no attempt to prolong the life of the microbes. In Series 2, the yeast was mixed daily in the feed with the aid of a small amount of water. Lyophilized yeast was used in Series 3. Daily additions of yeast were made in the experiments with poults (Series 4). The basal ration used in Series 1 and 2 was a practical type of chick ration (Table I). The ration in Series 3 was of a purified type which contained methanol-extracted soybean oil meal as the protein source(6). A practical poult-starting ration was used for the fourth series of experiments (Table I). The APF supplement which was used in some trials contained 7 mg of aureomycin per g.

TABLE I. Basal Rations Used in Experiments with Chicks and Poults.

Ingredient	Series 1 & 2 (chicks), %	Series 4 (poults), %
Corn, yellow, ground	30	20
Wheat, ground	13.5	10
Barley ”	10	13.8
Alfalfa meal, dehydrated	4	5
Wheat mill run	15	10
Soybean oil meal	10	25
Fish meal	7.5	7.5
Meat scrap		2.5
Liver meal	2	
Dried whey	2.5	2
Dried skim milk	2.5	
Ground limestone	1.5	2
Bone meal, special steamed	1.2	1.5
Salt	.5	.5
Mn sulfate	.025	.025
Feeding oil (2250 A, 300 D)	.25	
Dry ‘‘A’’ (10000 units/g)		.075
” ‘‘D’’ (2000 ”)		.01
Riboflavin	1 mg/lb	
Butyl fermentation product		.25

Results. In the first series of chick tests (Table II) there was no response to yeast G-1-B in either the presence or absence of streptomycin. Growth was slightly reduced by yeast E-16 alone, but its addition to the ration containing streptomycin produced a slight gain. In these trials the feed was mixed only at the start of the experiment.

In an attempt to keep the yeast viable for a longer period, daily mixing was used in the second series (Table III). In Exp. 6, there was a slight growth increase with all of the yeasts tested. Since some of this may have been due to stimulation in feed consumption by water added to the feed, in the remaining trials control groups were included with water added in amounts equivalent to that used in mixing the yeast in the feed. Yeast E-16 gave a very slight increase in growth above the water-mixed control in 2 experiments. Pasteurizing and lyophilizing had little effect upon its growth-promoting activity.

In Series 3 (Table 4), yeast E-16 was lyophilized and mixed in the feed at the start of the experiment at a level equivalent to 2% of the moist yeast. The results show a slight increase in percentage gains for 2 of 3 trials.

[‡] We are indebted to Mr. Sidney Saperstein and Professor Herbert G. Johnstone for aid in this matter.

TABLE II (Series 1). Effect of Antibiotics (50 mg/kg), APF Supplement (.25%) and Yeast (1%) upon Growth of Chicks.
(Feed mixed at start of experiment only.)

Supplement		Avg gain (g) Exp. 1	Exp. 2	Exp. 3*	Exp. 4*	Exp. 5*
0		205	237	130	253	195
Streptomycin		220	223	124		
APF supplement				136	259	214
Yeast	E-16		204	121†	244	
	E-16 (lyophilized)			129		
	G-1-B	203			231	198
Brewer's yeast				129		
Streptomycin + yeast	E-16		249			
	G-1-B	222				
APF supplement + yeast G-1-B						227
No. of chicks/group		15	20	20	20	20
Duration, days		22	20	21	28	28

* Avg of male and female gains.

† Chicks inoculated with yeast E-16 orally at start of exp., none in feed.

TABLE III (Series 2). Effect of Antibiotic (50 mg/kg), APF Supplement (.25%) and Yeast (1%) upon Growth of Chicks.
(Feed mixed daily.)

Supplement		Avg gain (g) Exp. 6*	Exp. 7*	Exp. 8	Exp. 9*
0		202	263		130
					124
Water			275	218	133
Aureomycin		200		267	
APF					136
Streptomycin		218	279	239	
Yeast E-1		211			
Yeast E-16		217	283	229	129
	L†				127
	P				137
	L & P				122
Yeast E-34		213			
" G-1-B		220			
Streptomycin+E-1		236			
" +E-16		224			
APF + yeast E-16					144
No. of chicks/group		20	20	28	20
Duration, days		20	22	28	28

* Avg of male and female gains.

† L = lyophilized; P = pasteurized.

The difference, however, was not great enough to be statistically significant even at the 5% level. Liver concentrate increased growth consistently, and yeast added to it caused no further increase in growth. The response due to aureomycin was slightly less than was required for significance at the 5% level. Yeast and liver concentrate gave increases which were not statistically significant when added to the aureomycin-fed groups.

In the 4th series, yeast E-16 was tested with poults (Table V). In 2 trials it failed to increase growth above the control ration which was mixed with water daily.

Discussion. The mechanism through which antibiotics accelerate the early growth of chicks and poults is obscure. Although there are many changes in the microflora of the intestinal tract when antibiotics are fed, the observation that the yeast population increased was of particular interest. This information, coupled with the fact that under certain conditions a commercial live yeast culture had been noted to increase growth in chicks pointed to the possibility that antibiotics might owe their activity to the stimulation of intestinal yeast growth. It might be postulated that the yeast supplied a nutrient essential for chicks and poults. Although the birds fed yeast showed a slight increase in some trials, antibiotics had an even greater and more consistent effect. Testing of the hypothesis regarding a possible role of intestinal yeast in the antibiotic growth effect is far from completed; however, the present results suggest that the postulate is incorrect.

These experiments were started before the relative value of the different antibiotics in promoting growth had been established. In previous work(4), streptomycin was shown to give a definite growth response, although it was less than that obtained with penicillin. McGinnis(7) has shown that streptomycin was relatively ineffective when fed at 5 mg per kilo of feed but at 25 mg per kilo it pro-

TABLE IV (Series 3).
Effect of Lyophilized Yeast and Liver Concentrate upon Growth of Chicks.

Supplement level		Avg daily gain (%)							
		Without aureomycin				Plus aureomycin			
	%	Exp. 10	Exp. 11	Exp. 12	Avg	Exp. 10	Exp. 11	Exp. 12	Avg
0		6.28	6.12	6.03	6.14	6.45	6.34	6.17	6.32
Lyophilized yeast	2	6.32	6.40	5.94	6.22	6.44	6.40	6.39	6.41
Liver concentrated	.5	6.48	6.37	6.22	6.36	6.59	6.49	6.35	6.48
Lyophilized yeast + liver concentrate	2 .5	6.24	6.50	6.28	6.34	6.36	6.35	6.21	6.31

Least difference for significance at 5% level, .21 and at 1% level, .30.

TABLE V (Series 4). Effect of Antibiotic and Yeast Supplements upon Growth of Poults.

Supplement	Level	Avg of ♂ and ♀ gains (g) Exp. 13	Exp. 14
0		434	659
Streptomycin	50 mg/kg	464	
Procaine penicillin	50 "	538	669
Yeast E-16	1%	433	640
Streptomycin + yeast E-16	50 mg/kg 1%	502	
No./group		17	13
Duration, days		28	31

duced gains equal to aureomycin and terramycin. The level of streptomycin used here should have been adequate to give its maximum growth response.

The few strains of yeast isolated for these trials represent only a minute segment of the total microbial population; hence the present study does not provide conclusive evidence that alterations in the yeast or other microbial flora are not responsible for the antibiotic growth increase.

Summary. A 5- to 10-fold increase was noted in the yeast population of the intestinal contents of turkey poults when they were fed a ration containing streptomycin. When the yeasts were isolated and fed to other poults and chicks there was no consistent increase in growth, although very slight increases were noted in some trials. The growth increase observed with the feeding of antibiotics apparently cannot be simulated by feeding the particular strains of yeast isolated.

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Induction of Precocious Opening of Immature Rat Vagina with Neostigmine.[*] (19862)

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It has been possible to affect the genital cycle of adult polyestrous animals and to reestablish function in senile females by various drugs which act on the autonomic nervous system, but it has been generally held that the ovaries of immature rats or mice cannot be influenced in this manner(1-4). This report deals with the induction of precocious ovarian activity with consequent opening of the vaginal introitus of immature rats following the administration of the parasympathetic stimulant neostigmine.

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[*] My thanks are due to Mr. V. Montesclaros for his efficient and faithful technical assistance, and to Hoffmann-La Roche Inc. for the Prostigmine used in this study.

Methods. A total of 106 immature rats of the Sprague-Dawley strain were employed. A group of 23 normal untreated animals in the colony were observed for the day of spontaneous vaginal opening. Twenty-eight rats 21 days of age at the beginning of the experiment received 0.5 cc (.025 g) of a solution of neostigmine (Prostigmine-Hoffmann-La Roche) subcutaneously 3 times daily, except on intervening Sundays, and were sacrificed in small groups at various times from age 26 to 56 days; a control group of 30 were similarly treated using plain water for injection. Five rats age 21 days, with 5 controls, were given injections of the same dose of neostigmine twice daily for 20 days. Six rats were castrated at age 21 days and injected 3 times daily for 15 days with neostigmine. The hypophysectomized animals were of the same strain but were obtained from a commercial laboratory. They were operated upon on day 21, and injections were begun at 30 days of age. An attempt was made to give the same high dosage of neostigmine but this was impossible owing to toxic effects and they actually only received a total equivalent to 2 injections of .025 g daily. Of these hypophysectomized rats only 3 experimental and 6 control survived treatment for 16 days. In all experiments body, uterine and ovarian weights were recorded at autopsy. The ovaries, uteri, and vaginas were fixed in Bouin's solution for section and microscopic examination.

Results. The 23 untreated animals of our colony showed that vaginal opening normally occurs at from 37 to 45 days inclusive, with 41 days as the mean.

The effect on vaginal opening of the injection of neostigmine 3 times daily as described above, is shown in the accompanying illustration (Fig. 1). Except for 4 rats autopsied at 26 days, the vaginas of all the experimental animals opened at times varying from 29 to 35 days. The vaginas of 18 control rats autopsied before age 36 days were closed, and opening did not occur until 37 to 41 days in the 12 observed for longer periods of time.

Age	Expt'l	Controls
41		oo
40		
39		oo
38		oo
37		oooooo
36		
35	o	xxxxxx
34	oooo	xxx
33	oooo	xxx
32		
31		x
30	oooooooo	
29	ooooooo	
28		
27		
26	xxxx	xxxxx
25		

o=Day vaginal opening
x=Closed at autopsy

FIG. 1. Each symbol (○ or ×) represents one animal. Vaginal opening occurred at 29 to 35 days of age in all the experimental animals except those autopsied at 26 days. In the control group vaginal opening was noted at from 37 to 41 days of age.

The toxic effect of neostigmine was apparent in lesser body weight, and ovarian weights were also less except in the rats observed long enough for cyclic ovarian function to have become established. The histologic examination of the ovaries of the experimental rats did not show any abnormal picture, but in the group under 35 days of age with opened vaginas a more advanced stage of ovarian development over the controls was discernible. This was seen in the presence of larger and more mature follicles, luteinization, and a marked development and hypertrophy of interstitial tissue both of thecal and germinal epithelial origin.

The importance of the dosage of neostigmine required for this effect is seen in the fact that the vaginas of the rats injected only twice daily opened one day later (39 days) than those of their controls. Also of interest, if not significant, is the observation that 2 Sundays with no injections intervened in the group with vaginal opening at 33 days, and with those at 34 and 35 days there was a long holiday weekend, again with a diminution in the number of injections. On the other hand, only one Sunday intervened in the groups with vaginal opening at 29 and 30 days.

The necessary involvement of ovarian function to produce vaginal opening is indicated not only by the histological findings given above but by the fact that it was not achieved after 15 days of treatment 3 times daily in 6 immature castrates.

Since it was not possible to give adequate amounts of neostigmine to the hypophysectomized rats used in this study, the question of anterior pituitary involvement in this regard remains unsettled. Other workers(5), however, have been able to stimulate ovarian function in normal adults by direct application of this drug to the hypophysis.

Summary. The daily subcutaneous administration of adequate doses of neostigmine to immature rats has resulted in a premature development of ovarian function with a precocious opening of the vagina.

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Plasma "Thrombocytopenic Factor" as Measured by Direct and Indirect Platelet Methods.* (19863)

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A prompt decrease in the blood platelets as measured by the indirect method occurred in 8 of 10 human subjects following the intravenous administration of normal human plasma. However, no decrease was observed when the platelets were evaluated by the direct citrate method.

Harrington *et al.*(1) demonstrated a "thrombocytopenic factor" in the blood and plasma of patients with idiopathic thrombocytopenic purpura (ITP) but observed no such effect of normal whole blood. Stefanini and Chatterjea(2) reported a temporary but significant decrease in platelets in 32 of 36 subjects when compatible blood plasma or serum from non-thrombocytopenic donors was transfused. These investigators measured the platelets by the indirect method of Dameshek(3). We previously confirmed the observations of Harrington *et al.* as to the thrombocytopenic effect of plasma from subjects with ITP as measured by the indirect method but observed no decrease in platelets as measured by the direct citrate method(4). The effect of normal plasma was not studied.

Materials and methods. Fresh pooled normal plasma was obtained from the blood of normal donors. The blood was collected in acid citrate dextrose solution. Irradiated pooled human plasma was obtained commercially in the lyophilized state. Dextrose solution, 5% in H_2O, was used as a control to be certain that the platelet decrease was not due to a speed-shock reaction. See Table I for recipient data. No transfusion reactions occurred. All intravenous fluids were administered within 30 minutes. Platelets were counted at intervals (Fig. 1, 2) by the direct citrate method of Rees and Ecker(5) and the indirect method of Dameshek(3). The following platelet levels were considered normal: Rees and Ecker method, 225000 to 350000 per cu mm; Dameshek method, 500000 to 900000 per cu mm.

Results. a) *Effect of intravenous 5% dextrose in H_2O.* The two subjects were observed. There was no effect on the platelet levels as measured by either the direct or indirect methods.

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TABLE I. Data on 12 Subjects Receiving Transfusions of Pooled Plasma or Dextrose Solution.

Age	Sex	Diagnosis	Type of transfusion	cc administered
36	♀	Common duct obstruction	Fresh plasma	210
60	♂	Carcinoma of stomach		210
18	♀	Anxiety neurosis		350
39	♂	Metastatic carcinoma of liver		350
62	♂	Cirrhosis of liver		200
42	♀	” ” ”		300
57	♂	Duodenal ulcer (uncomplicated)		210
68	♂	Lobar pneumonia	Irradiated plasma	250
34	♂	Epilepsy		
64	♂	Emphysema		
22	♂	Epilepsy	5% dextrose in H_2O	
49	♂	Psychoneurosis		

b) *Effect of fresh pooled normal human plasma.* Seven recipients (subjects 1 to 7, Table I) were transfused with fresh normal human plasma. In 5 subjects there was a significant decrease in the circulating platelets as measured by the indirect method (Fig. 1). The thrombocytopenic effect was transient. No drop was noted in the platelets as measured by the direct method (Fig. 2).

c) *Effect of pooled irradiated lyophilized human plasma.* Three recipients were given pooled irradiated human plasma (subjects 8 to 10, Table I). Again the decrease in platelets as measured by the indirect method was noted (Fig. 1). No fall occurred in the platelet levels as measured by the direct method (Fig. 2).

Discussion. Apparently there is a "thrombocytopenic factor" in human plasma. It is present in the globulin fraction(1), stable at 56°C, not absorbed by either Seitz filters or ion-exchange resins, but is absorbed on $Ca_3(PO_4)_2$ gel(2). Blood or plasma from patients with ITP produces a prolonged de-

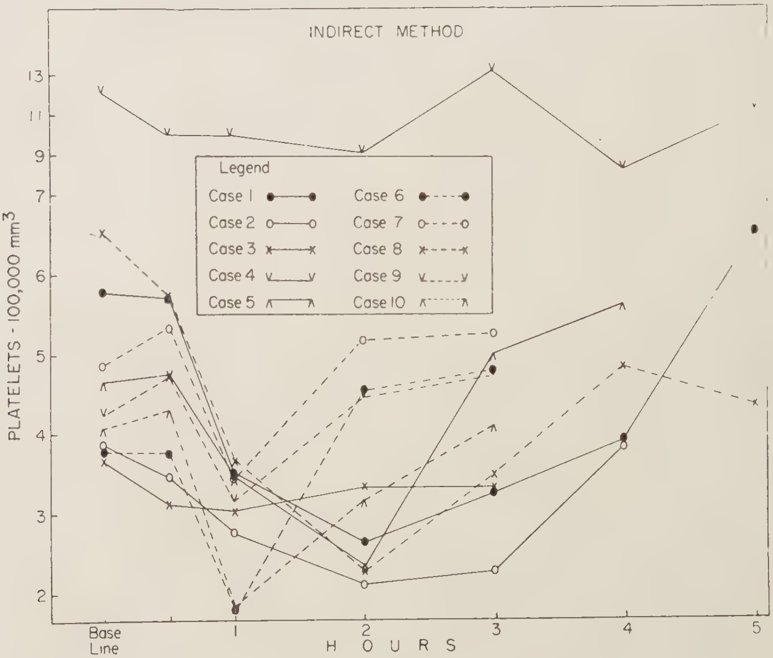

FIG. 1. Effect of pooled normal plasma transfusions on platelet counts by the indirect (Dameshek) method.

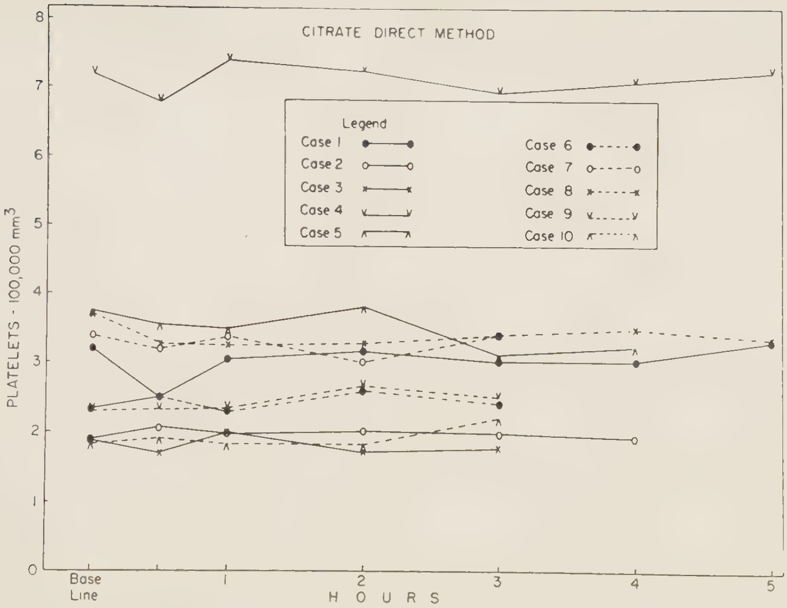

FIG. 2. Platelet values (direct method, Rees and Ecker) following transfusions with pooled normal plasma. Observations simultaneous with those in Fig. 1.

crease in platelets as measured by the indirect method(1,4). In our series(4) the most rapid return to normal occurred in 24 hours (1 case). In other subjects the decreased platelet level continued over a period of several days(1,4). The present study on the effect of normal plasma on platelets confirms the observations of Stefanini and Chatterjea(2) but is in disagreement with the observations of Harrington *et al.*(1). The decrease in platelets following the administration of normal plasma is rapid and transient, and not as prolonged as that following the transfusion of ITP plasma.

There is a tremendous variation in platelet values obtained by the direct and indirect technics. Wintrobe(6) states that incorrect high platelet levels can be caused by fragmentation without dissolution. Tocantins(7) reported that it would seem that figures above 500,000 per cu mm are seldom, if ever, obtained in cutaneous blood under normal conditions, and are perhaps the result of a defective technic. As we have previously stated (4) it is theoretically possible that the "thrombocytopenic factor" is a platelet-fragmentation inhibiting substance that can best be measured by correlating platelet values obtained by both direct and indirect methods. Our present studies show that it is present in both normal and ITP plasma. The basic difference in the two technics is that in the direct method there is a greater dilution of the blood (1:200) and in the indirect method the dilution is less (1:5). Is the substance causing platelet-fragmentation diluted by the direct method to the extent that most of its mechanism of action is inhibited?

Conclusions. A temporary and significant decrease in platelets as measured by the indirect method occurred in 8 of 10 individuals following the transfusion of normal blood plasma. No decrease in platelets occurred as determined by the direct method of evaluating platelet levels. The possible cause for the differences in the results obtained by the direct and indirect methods is discussed.

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Lack of Correlation of Eosinopenic and Adrenal Ascorbic Acid-Depleting Activities of Various Adrenocorticotropic Preparations.* (19864)

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Evidence has been presented(1-3) suggesting that eosinopenic activity (EA) is not directly correlated with the adrenal ascorbic acid-depleting activity (AAA) of various ACTH preparations. The following experiments present additional evidence that EA and AAA are not directly correlated, and further, that marked reduction of the AAA of ACTH preparations as produced by alkali-heat treatment does not proportionately inactivate the EA.

Methods. Normal and hypophysectomized male rats of the Long-Evans strain, 60-80 days of age, were employed. The hypophysectomized animals were used for eosinophil counts during the first 7 postoperative days, the animals being used repeatedly at daily intervals. Normal animals were reused at weekly intervals. Direct eosinophil counts were taken from tail vein blood using the diluent described by Speirs and Meyer(4). The blood was diluted 1:20, and eosinophil counts were made on two chambers of a Fuchs-Rosenthal hemocytometer (0.2 mm deep). An initial count was taken, followed immediately by intraperitoneal injection of the solution to be tested and a second count was taken four hours later. All animals with initial eosinophil counts lower than 100/mm^3 were discarded. Care was taken to control environmental and temporal variables in order that data from all experimental groups could be directly compared. Adrenal ascorbic acid-depletion activity in hypophysectomized rats was determined for each ACTH preparation by the method of Sayers *et al*(5) and all activities are expressed in terms of the U.S.P. standard. The ACTH preparations tested were prepared by methods previously described, a reference number for each preparation being given in Tables I and II. In addition, two ACTH preparations, L 2317 M and L 2337 M, were each treated with either HCl or NaOH as follows: a) ACTH was dissolved in 0.1 N NaOH, heated immediately on the boiling water bath for 10 minutes, immediately brought to pH 5 with HCl, and diluted to volume with 0.9%, *i.e.*, NaCl, and b) ACTH was dissolved in 0.2 M HCl, refluxed for 3 hours on the boiling water bath, brought to pH 5 with NaOH, and diluted to volume with 0.9% NaCl.

Results. The data presented in Table I for the normal rat show that ACTH preparations of widely different AAA may exhibit EA of such varying degrees as to suggest the existence of an inverse relationship between AAA and EA. On the other hand, the results for the same preparations assayed for EA in the hypophysectomized rats show (Table I) even greater discrepancies between EA and AAA suggesting that non-specific responses in normal rats may completely vitiate conclusions drawn from eosinophil assay of ACTH in normal rats.

Table II summarizes data which demonstrate that the treatment of ACTH preparations with alkali-heat under the prescribed conditions markedly reduces the AAA of the ACTH preparations, but does not alter the EA in hypophysectomized rats at the dose level tested. In order to demonstrate that the 2.0 mg dose level of NaOH treated-ACTH was not so excessive as to mask a reduction of EA of this preparation, the original material (L 2337 M) was tested in a separate group at the 0.1 mg level which was calculated to approximate the AAA unitage of the NaOH-heat treated ACTH preparation. It was found that

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TABLE I. Comparison of Eosinopenic and Adrenal Ascorbic Acid-Depleting Activity (AAA) of Various ACTH Preparations in Normal (N) or Hypophysectomized (H) Rats.

Preparation tested*	Reference No.	No. of rats	Type of rat	Mean† eosinophil change, % ± S.E.	Estimated AAA depleting activity, IU/mg
Control groups					
Saline		62	N	—17 ± 5	
		46	H		
Exp. ACTH groups					
L 2262 A	8	13	N	—66 ± 7	.2
		22	H	—31 ± 9	
2010 M	7	13	N	—53 ± 9	1
2262 B	8	9	N	—49 ± 9	30
2269 B	8	4	N	—39 ±14	120
		5	H	+79 ±36	
2170 C	9	5	H	—13 ± 7	7

* All preparations tested at 1 mg dose level after single intrap. inj. except L 2170 C which was tested at the 5 mg level.

† The mean initial eosinophil count of 50 normal rats chosen at random from the groups above was 270 ± 28 cells/mm^3, of 26 hypophysectomized rats on post-operative day 1: 518 ± 56, day 2; 475 ± 45, day 3; 557 ± 61, day 6; 545 ± 57.

TABLE II. Comparison of Eosinopenic and Adrenal Ascorbic Acid-Depleting Activity (AAA) of Alkali- and Acid-Treated ACTH Preparations in Hypophysectomized Rats.

Preparations tested*	Reference No.	No. of rats	Mean eosinophil change, % ± S.E.	Estimated AAA depleting activity, IU/mg
Control groups				
Saline		46	— 5 ± 4	
Growth hormone		5	— 5 ±14	
Exp. ACTH groups				
L 2317 M	8			
Untreated		9	—66 ± 4	60
NaOH treated		13	—54 ± 6	2
HCl "		8	—56 ± 4	60
L 2337 M	8			
Untreated		9	—76 ± 8	40
" †		7	—25 ± 4	
NaOH treated		9	—63 ± 9	2
HCl "		4	—77 ± 4	40

* All preparations tested at 2 mg dose level after single intrap. inj. except (†) which was tested at the .1 mg level.

at this equivalent unitage level, a distinctly lower EA was found in the untreated material, suggesting that a masking of the EA of the NaOH-heat treated material had not occurred at the 2.0 mg dose level. It is of interest that the HCl-heat treatment of the ACTH as described did not significantly alter the EA of the preparations employed.

Discussion. Evidence has been presented to indicate that there is a lack of correlation between EA and AAA of ACTH preparations assayed in normal rats. An even greater lack of correlation is noted when the same preparations are tested in both normal and hypophysectomized rats. Further, it is shown that alkali-heat treatment of ACTH preparations, under the conditions employed here, reduced the AAA(6), but not the EA of such preparations. It is of interest to note, in this connection, that it has been reported that ACTH is almost completely inactivated, as judged by the adrenal repair test in hypophysectomized rats, after treatment for 30 minutes in 0.1 N NaOH at 100°C(7). On the other hand, it has been shown(6) that treatment of ACTH in 0.1 N NaOH for 10 minutes in the boiling water bath causes approximately 90-95% inactivation of AAA, suggesting that alkali-heat inactivation of AAA proceeds in a manner independent of EA inactivation during this time period.

It must be emphasized that preparation L 2262 A is an unadsorbed fraction obtained from oxycellulose treatment(8). As shown in Table I, this fraction shows minimal AAA, yet retains some EA, as tested in both normal and hypophysectomized rats. This finding adds increased weight to the possibility that AAA and EA may not be identical.

It has previously been emphasized(2) that, since ACTH fractions lacking in AAA are usually discarded, it is not surprising that we have not found EA in the complete absence of AAA in any of the preparations tested. On the other hand, ACTH preparations highly active in AAA (L 2269 B) may exhibit no EA in hypophysectomized rats.

It is worthy of note that certain ACTH preparations are eosinophilic in action in normal mice and in hypophysectomized rats. An instance of this is recorded in Table I. It is also of note that the administration of 1 to 5 mg doses of ACTH to hypophysectomized rats has not duplicated the maximal depletion of eosinophils noted in hypophysectomized animals with hydrocortisone (–95% on the average for dose levels of 1.0 mg or more)(11). Such a discrepancy between an ACTH induced eosinophilia, and the failure of large doses of ACTH to duplicate in degree the effects of hydrocortisone carries the implication that an eosinophilic component may be present in the ACTH preparations, thus blocking or masking EA. It remains to be shown, however, that this eosinophilia in the hypophysectomized animal is not a non-specific protein effect. Although growth hormone was used as a control material, without effect on the eosinophil level in the hypophysectomized rat, it is felt that still other inert proteins must be tested for possible eosinophilic activity at comparable dose levels. A further possibility exists that certain ACTH preparations may cause the secretion of such small amounts of eosinopenic steroids as to cause an eosinophilia. It has been reported, for example, that in adrenalectomized mice, doses of cortisone acetate in amounts smaller than 1 μg may cause an eosinophilia(10), and in this laboratory, cortisone acetate at a dose level of 250 μg has been found to cause an eosinophilia in hypophysectomized rats(11).

Summary. Equivalent dose levels by weight of various ACTH preparations have been compared on the basis of their eosinopenic activity (EA) in normal and hypophysectomized rats with the adrenal ascorbic acid-depleting activity (AAA) in hypophysectomized rats. The data presented have been interpreted as indicating that: 1. No correlation exists between the EA and AAA of various ACTH fractions as tested either in normal or hypophysectomized rats. 2. EA in normal rats is not necessarily correlated with EA of the same preparations as tested in hypophysectomized rats. 3. Reduction of AAA by treatment with NaOH-heat under the conditions employed does not inactivate EA, suggesting that EA may be a separate and more stable component of the ACTH preparations. Neither AAA nor EA were influenced by HCl-heat treatment in these experiments. 4. No ACTH compound has been tested which is completely free of AAA and yet exhibits EA, although the converse has been observed. 5. Some evidence suggests the presence of an eosinophilic component in certain ACTH preparations.

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Normal Iodine Uptake and Anoxia Resistance Accompanying Apparent Hypometabolism in Hereditary Obese-Hyperglycemic Syndrome.*† (19865)

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The hereditary obese-hyperglycemic syndrome of mice is a recessive Mendelian entity characterized by an adult weight up to 3 to 4 times the normal, sterility(1), high blood sugar levels and glucosuria associated with insulin resistance(2), atrophy and ulcers of the skin, enlarged islets of Langerhans(3), increased food intake and modified food choices (4), decreased spontaneous activity(5), relatively low oxygen consumption(6), and a number of atypical reactions to hormonal and dietary treatments(7). The obese animals also show decreased oxidation of administered acetate and an increased conversion of acetate to fatty acids(8). The possible relation of hyperglycemia to a primary biochemical block has been discussed(6). Its etiological significance as regards hyperphagia has also been examined(9,10). Of special relevance to the study reported here are the following observations: the total basal oxygen consumption of obese mice is not higher than that of the non-obese animals, in spite of the fact that they weigh twice or 3 times the non-obese average(6). On a body surface basis, the basal metabolic rate of the obese animals can be calculated as 40 to 50% below normal. The low oxygen consumption of the obese animals is accompanied by normal fasting respiratory quotients, somewhat elevated non-fasting respiratory quotients(7). Obese animals are unusually sensitive to thyroxine from the standpoints of increased oxygen consumption, weight loss and toxicity(7). Serum and tissue cholesterol levels are high(6,7). As noted above, there is decreased spontaneous activity(5). These findings could be construed as suggesting hypothyroidism. On the other hand, the histological picture of the thyroid of obese animals is normal(3). Administration of thiouracil to non-obese animals, although it decreases their oxygen consumption, fails to reproduce any part of the obese-hyperglycemic syndrome(7). Finally, neither hyperphagia nor insulin resistance are generally associated with hypothyroidism.

Because of the conflicting lines of interpretation with respect to thyroid function in these obese mice, it appeared of importance to directly assess the physiological status of the thyroid by determination of radioactive iodine uptake. It also seemed of interest to compare metabolic rates of obese and non-obese animals on a basis more independent of body size. The propriety of comparing metabolic rates on a body surface basis when dealing with animals of widely different fat content has been questioned in a previous study(6). The anoxia method(11-14) which compares the relative degree of dependence of animals on oxygen tension has been shown to be de-

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† The animals used in this study were obtained, as in previous experiments, through the kindness of Miss Margaret M. Dickie of the Jackson Memorial Laboratory, Bar Harbor, Me.

TABLE I. Uptake of Radioactive Iodine by the Thyroid Gland of Obese and Non-Obese Mice.

Exp. No.	Exp. animal	Body wt	Interval after inj. (hr)	I^{131} uptake (% of inj. dose)
1	Non-obese (7)	20-30	18	4.7±1.1
	Obese (7)	38-58		5.7±1.6
2	Non-obese (7)	22-28	30	14.8±5
	Obese (7)	28-50		12 ±2.2

Figures after ± signs are stand. dev.

pendent on metabolic rates. For example, thyroxine decreases, while thiouracil increases the resistance to anoxia of normal animals.

Methods and results. Fourteen young obese animals, and 14 controls of the same age and sex distribution were used in the iodine uptake determinations. The animals had been maintained on a diet of Purina Lab Chow, in individual cages at constant temperature. All animals were given a single intraperitoneal injection of one microcurie of carrier-free I^{131}. The mice were kept in raised cages to prevent access to feces. Eighteen or 30 hours after injection, the mice were anesthetized with ether, exsanguinated, and the thyroid glands were carefully dissected out and freed of extraneous tissue with the aid of a binocular microscope. The glands were digested in 1 ml 2N NaOH, diluted to a convenient volume, and aliquots were counted with a thin mica end window Geiger-Muller counter.

The uptake of radioactive iodine by the thyroids of the obese and non-obese mice is shown in Table I. It will be seen that there is no significant difference in the radioactive uptake of the thyroids of the two groups of mice.

Ten animals, 5 obese and 5 non-obese of the same age and sex distribution were used in the anoxia test. They were confined in a closed container (desiccator). The carbon dioxide produced was fixed by a solution of concentrated alkali. All animals, whether obese or non-obese, died between 92 and 100 minutes after the beginning of the experiment. Within this interval, no difference in duration of survival attributable to obesity could be observed. Average survival was 96 minutes for both groups.

Discussion. The results of the radioiodine uptake experiment constitute additional and fairly conclusive confirmation of the normalcy of thyroid function in these obese animals. The anoxia test, similarly, suggests that active tissues, in both obese and non-obese animals, are equally dependent on oxygen tension and have presumably comparable metabolic rates. These results therefore confirm histological evidence(3) as well as the interpretation of the negative results of thiouracil administration to non-obese animals(7). High cholesterol levels are difficult to interpret in the presence of diabetic-like conditions. Sensitivity to thyroxin is not necessarily related to hypothyroidism.

Finally, the results of the anoxia test bring additional evidence to support the contention that relating metabolic rates to either body weight or body surface when comparing obese and non-obese animals is meaningless. The protein and water contents of obese and non-obese are similar. The excess weight carried by obese mice can be accounted for to the extent of about 90% by fat(15). The fact that this increase in body weight or in body surface is accompanied by no increase in oxygen consumption, and that the anoxia test indicates that active tissues have comparable metabolic intensities in both groups, simply testifies to the almost complete metabolic inertia of depot fat in this syndrome.

Summary. In the hereditary obese hyperglycemic syndrome of mice, radioactive iodine uptake is normal. Similarly, the anoxia test reveals no difference in dependence on oxygen tension between obese and non-obese mice. The significance of these findings is discussed with respect to the etiology of the syndrome, and to the extension of the concept of basal metabolic rate to obese animals.

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Application of Perfused Rat Liver Preparation to Isotopic Study. (19866)

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It seems a reasonable assumption that an isolated liver forming bile is a living liver. Such a preparation lends itself to many useful studies. Many apparatus and methods for perfusing the isolated liver of the rat have been used. A particularly good method emphasizing bile production has been developed by Brauer and coworkers(1). The authors have presented evidence, in addition to bile production, that the liver is viable during perfusion. This recent paper appeared subsequent to the completion of the present study. A perfusion apparatus that performs reasonably satisfactory has been made in this laboratory and adapted to the investigation of liver physiology in relation to radioactive materials. Gallium^{72} citrate was used in preliminary experiments.

General perfusion technic. The perfusion model, designed by one of the authors (M.B.), was fashioned in the shop of this laboratory from lucite. The essential parts comprising the apparatus are shown Fig. 1. They consist essentially of an oxygenation chamber, an artificial heart, a windkessel, which acts to convert a pulsatile into a constant flow, and an adjustable side reservoir. Polyethylene tubing is used for connections. About 90 ml of blood are required for operation of the present model. The artificial heart is driven by a variable stroke pump[†] set to deliver about 110 ml of air per stroke at a stroke rate of about 190 per minute. Blood flow from the portal vein cannula, when attached separately to the apparatus, has been measured as about 7.5 ml per minute. However, blood flow from the inferior vena cava during perfusion has been measured as about 2.5 ml per minute. The flow from the portal vein cannula is delivered under a head of constant

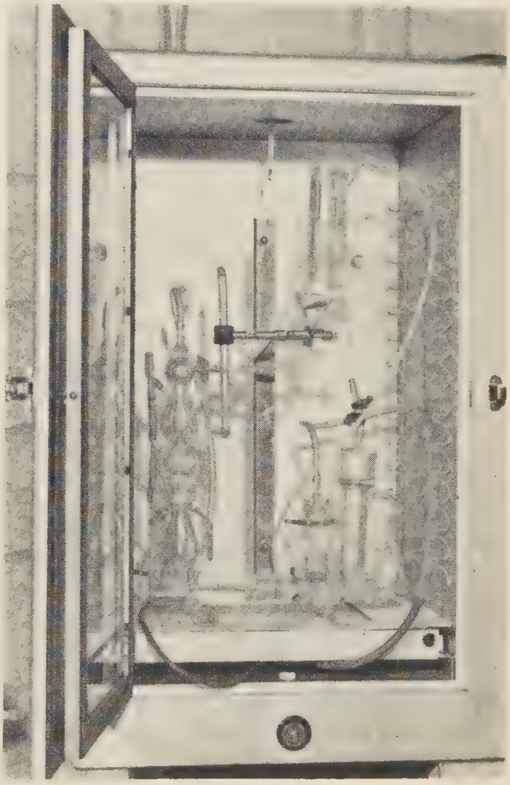

FIG. 1.

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[†] C. F. Palmer (London) Ltd.

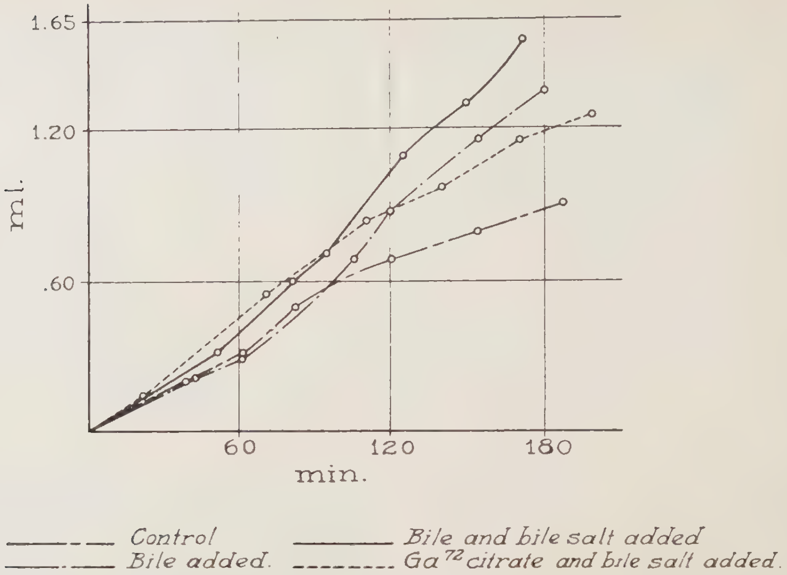

FIG. 2. Bile flow as evidence of viability in liver perfused with solution containing .26 millicurie of Gallium^{72}.

pressure of approximately 10 in. of water. A mixture of 95% oxygen and 5% carbon dioxide is supplied the oxygenation chamber at the rate of about 2 liters a minute. The gas mixture is passed through water before entering the oxygenation chamber. Temperature of the heating cabinet in which the perfusion machine is placed is kept at 37° to 38°C. The blood used is heparinized fresh whole rat blood. A typical perfusion experiment is carried out as follows. A large adult rat (Wistar), preferably male, is anesthetized with sodium pentobarbital, the abdomen opened, and the blood heparinized. The bile duct is cannulated with polyethylene tubing (i.d. .019″) by approaching it from the duodenum. The inferior vena cava is then cannulated with a short polyethylene cannula, preferably slightly larger than that used in the portal vein. This cannula is stoppered. Lateral vessels (usually 2) of the portal vein, just before entrance into the liver, are tied. The portal vein is then cannulated with tubing (i.d. .062″) which has been carefully filled with blood. It is very important that no air be in this cannula. This cannula is also stoppered. The perfusion apparatus is removed from the heating cabinet while cannulation of the portal vein is proceeding. Circulation of the blood in the apparatus is begun, and the portal vein cannula is then unstoppered and attached as quickly as possible. The stopper is removed from the inferior vena cava. The liver is cut out of the animal and placed in the organ receptacle with the inferior vena cava cannula pointing downward. The interval elapsing from the time the portal vein is cannulated to the time the liver is finally installed is about 2½ minutes. The apparatus is then placed back in the heating cabinet and bile flow begins almost immediately.

Gallium^{72} citrate. The present study was conducted primarily to demonstrate the feasibility of using a radioactive material with the isolated rat liver perfusion system with bile production. Gallium^{72} citrate has been used. In Fig. 2 are presented the curves of bile production plotted from the data of 4 experiments, in each of which a separate rat liver was used. Bile and dehydrocholic acid were added not primarily to measure volume of bile flow as affected by these agents but rather to enhance bile flow which furnished evidence for viability of the liver.

Four curves of bile flow are shown in Fig. 2. They are as follows: 1) Nothing was added to the perfusing blood (control); 2) 0.75 ml of perfused bile and 1.14 ml of bile

TABLE I. Activity of Blood, Liver, and Bile in Perfused Rat Liver Preparation.

Sample	Counts/min/ml or g ($\times 10^4$)
Blood (30 min intervals)	
1	8.5
2	8.4
3	7.7
4	8.5
5	8.5
6	8
Liver	2.2
Bile	1.8

obtained from another rat *in vivo* were added; 3) 0.60 ml of perfused bile plus 0.9 mg of dehydrocholic acid were added; and 4) 0.5 ml of gallium citrate solution, containing Gallium^{72},‡ and 0.5 mg of dehydrocholic acid were added. The latter solution contained 0.26 millicurie of Gallium^{72} activity. The quantity of gallium metal in the gallium citrate solution usually prepared was 8 to 10 mg per ml. All substances were added by way of the side reservoir.

Each perfusion was carried out about 3 hours, as shown in Fig. 2. Brauer and coworkers have obtained maximum rate of bile flow in this early phase(1). The bile and dehydrocholic acid were added at various times throughout the course of the perfusion.

The gallium citrate solution, containing Gallium^{72} was introduced into the perfusing blood 20 minutes after perfusion had begun. In this experiment samples of blood (0.1 ml) were taken every half hour for radioactivity measurement. The liver was perfused with isotonic saline several times and then digested with concentrated nitric acid. A diluted sample was placed in a small Petri dish for counting. The diluted samples of blood and bile were similarly counted. A lead-walled, horizontal counting chamber with a thin-walled Geiger-Mueller tube was used. The activity is expressed as counts per minute, corrected to a zero time, per ml, or g (Table I).

Results. It is evident that the introduction of the 0.26 millicurie of Gallium^{72} and stable gallium citrate into the perfusing blood did not prevent the isolated liver from producing bile for a period of at least about 3 hours. On the assumption that the isolated liver producing bile is a living liver, it is concluded that the liver was viable throughout this time. It seems the curves presented in Fig. 2 indicate strongly that the volume of bile produced was not, at least, drastically modified for that length of time after the 0.26 millicurie of activity had been introduced.

The radioactivity measurements are presented in Table I. The level of activity in the blood throughout the 3 hours remained rather constant. The liver had considerably less activity than the blood and the bile slightly less than the liver. Thus, it seems evident the bile per unit volume contained nearly as much Gallium^{72} as the liver. These data indicate that the particulate state of the gallium or gallium compound was not of sufficient size to be very effectively retained by the reticuloendothelial system of the liver. Both the higher level of activity in the blood and the nearly equal levels of activity of the liver and bile seem to support this.

Conclusion. The technic of isolated rat liver perfusion with bile production seems to be quite applicable to the study of the effects of radioactive isotopes on certain aspects of liver function; 0.26 millicurie of Gallium^{72} citrate solution with stable gallium has been used in the present study. The liver remained viable within the interval specified in the experiment. Blood activity levels remained constant. Both liver and bile contained Gallium^{72} although in lesser quantities than the blood.

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‡ Prepared in the laboratory of the Medical Division from irradiated $Ga(NO_3)_3$ by Dr. J. D. Perkinson.

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Effect of Stress and Cortisone on Plasma Protein-Bound Iodine and Thyroxine Metabolism in Rats. (19867)

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Cortisone and adrenocorticotrophic hormone suppress the uptake of I^{131} by the thyroid gland of experimental animals(1,2,17) and human beings(3-5). These substances also reduce the concentration of the plasma protein-bound iodine (PBI)(3,4,6,7). Various types of stress also depress I^{131} uptake by the thyroid of experimental animals(8-11) probably by increasing the secretory activity of the adrenal cortex. It therefore appeared probable that stress would reduce the plasma PBI by the same mechanism.

This line of reasoning ignores the importance of the rate at which the thyroid hormone is removed from the blood as a factor in determining the concentration of the hormone in the plasma. The following experiments were designed to investigate the effects of cortisone and stress upon the plasma PBI and on the peripheral utilization of thyroxine.

Methods. Two groups of male Sprague Dawley rats were used in these experiments. One group was thyroidectomized surgically and, after 6 months, treated with I^{131} (100 millicuries intraperitoneally to each animal) to assure complete destruction of the thyroid gland. The other group was of similar age, but was untreated. The thyroidectomized animals received 20 μg of racemic crystalline thyroxine subcutaneously each day starting seven days before the experiments were begun. At least one week was permitted to elapse between experiments. Protein-bound iodines were determined on 0.5 ml of plasma from heparinized blood obtained from the tail, using a modification of the alkaline ash method of Barker, Humphrey and Soley(12). Values obtained on 0.5 ml of plasma were not significantly different from the results obtained on 1.0 ml of plasma. In order to minimize the effects of repeated bleeding, not more than 2 samples were taken from any animal during a 24-hour period. The animals were subjected to the various stresses in sequence, with at least one week between experiments. Each animal served as his own control during the experiments. This method of calculation decreases the variation and increases the significance of the results statistically; however, the general conclusions obtained are not altered if the means of the entire group before and after treatment are compared. Three types of stress were studied. Animals exposed to low temperatures were kept in individual cages in a cold room at 4°C. Exercise stress was induced by placing the animals in a tank of water at 27°C and forcing them to swim for 2 hours. The thyroidectomized, thyroxine-maintained animals were so exhausted that the swim was terminated after only 105 minutes. The intact rats were also starved for periods up to 72 hours.

Results. In Table I, it can be seen that exposure to the cold produces a significant reduction in the PBI within 15 hours in intact, fasted rats. The PBI falls still further at 24 hours, but at 72 hours has returned to essentially normal levels. In contrast to this, prolonged fasting *per se* fails to alter the PBI during the first 15 hours, and produces a gradual continuing fall at 24 and 72 hours. The data presented in the second part of Table I show that when a constant source of thyroxine is maintained in thyroidectomized animals by injection, exposure to the cold depresses the PBI. The depression is even greater than the maximum observed in the intact rats ($p < .001$). These data indicate that the reduction in PBI as a result of exposure to the cold is not a result of decreased secretion of thyroid hormone; on the contrary, the fact that the fall of PBI is greater in the thyroidectomized rats indicates that an increase in thyroxine secretion probably occurs in the intact rats exposed to cold.

Violent exercise greatly reduces the PBI of intact rats (Table II, No. 1 and 2). The

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TABLE I. Effects of Cold Stress on Plasma PBI of Rats.

	Duration of exp., hr	Treatment	PBI*	Change of PBI, 4°C vs. control	P
		A. 6 normal rats			
1.	0	Fed control	4.4 ± .29		
2.	15	4°C (fasted) Control "	3.9 ± .07 4.6 ± .15	— .7 ± .03	<.001
3.	24	4°C (fasted) Control "	3.1 ± .16 4 ± .13	— .9 ± .16	<.01
4.	72	4°C (fasted) Control "	4.6 ± .22 3.1 ± .27	+1.5 ± .55	<.05
B.	8 thyroidectomized rats maintained on 20 γ thyroxine subcut./day				
5.	0	Fed control	4.6 ± .30	—3.6 ± .52	<.001
6.	15	4°C (fed)	1 ± .55		

* Values throughout are expressed as mean ± stand. error of the mean $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

thyroidectomized rats received their daily injection of thyroxine between the first (preswim) sample (Table II, No. 5) and the second sample, taken after swimming (Table II, No. 6). Consequently, a rise of PBI occurred even after the swim. When this rise is compared with the rise occurring at a similar time interval after the injection of an identical amount of thyroxine without exercise stress (Table II, No. 3 vs No. 4), the degree of rise is found to be reduced by exercise. The effect of exercise is therefore to diminish the PBI even when it is maintained by an unvarying source of thyroxine.

The injection of a single dose of 12.5 mg of cortisone subcutaneously failed to alter the PBI of normal rats, but continued injection for three days greatly depressed the PBI (Table III, No. 1 and 2). In contrast to this, the injection of a similar dose of cortisone for three days greatly increased the PBI of thyroxine-maintained thyroidectomized rats (Table III, No. 3). Moreover, the administration of cortisone completely prevented the expected fall of PBI in thyroidectomized rats after a 15-hour exposure to the cold (Table III, No. 4).

Discussion. These data demonstrate that 3 types of stress (cold, exercise and starvation) reduce the plasma protein-bound iodine level in intact rats. This effect is not entirely a result of suppression of thyroid activity, since the PBI is depressed by cold and exercise in thyroidectomized rats maintained on injected thyroxine. Presumably, therefore, these stresses act, in part at least, by increasing the rate of metabolism and destruction of thyroxine. Indeed, in the case of cold stress the depression of the PBI is greater in thyroxine-maintained thyroidectomized animals than in intact rats, suggesting that the rate of secretion of thyroid hormone has been augmented by exposure of the animal to the cold. Moreover, after 72 hours in the cold, the animals have a normal PBI, suggesting that the accelerated activity of the thyroid gland has "caught up" with the tissue demands for thyroxine. This finding is in agreement with the evidence of numerous observers, that the activity of the thyroid is increased when the animal is in a cold environment(9,13,14). The apparent decrease of I^{131} uptake by the thyroid glands of animals exposed to a cold environment is probably an artefact, as pointed out by LeBlond *et al.*(9), resulting from an increased turnover rate of iodine in the hyperactive gland.

Cortisone, in contrast to stress, appears to reduce the rate of metabolism of thyroxine injected into thyroidectomized animals. The adrenal hormone, in fact, is capable of completely blocking the augmented rate of disappearance of thyroxine produced by cold stress. The mechanism of this effect is obscure but it may be related in some way to the ability of adrenal steroids to increase the metabolic rate under circumstances where alterations of thyroid function cannot have

TABLE II. Effect of Exercise (2 Hr Swimming) on Plasma PBI of Rats.

Treatment	PBI	Change of PBI		P
A. 6 normal rats				
1. Control	4.2 ± .34	−3.1 ± .12		<.001
2. Swimming	1 ± .35			
B. 8 thyroidectomized rats Rats received 20 μg of dl-thyroxine subcut. daily.			Diff. resulting from swim stress	
3. Control, before thyroxine inj. on day of exp.	2.6 ± .49	+6.9 ± .55	−2.3 ± .72	<.01
4. Control, 2 hr after thyroxine inj.	9.4 ± .41			
5. Exp., before thyroxine inj. on day of exp.	3.9 ± .15	+4.6 ± .47		
6. Exp., after thyroxine inj. and 2 hr of swimming	8.5 ± .46			

TABLE III. Effect of Cortisone on Rat Plasma PBI.

Treatment	Duration of exp.	N	Dose, mg	PBI		Change of PBI	P
				Initial	Final		
1. Normal, fed	4 hr	6	12.5	2.5 ± .45	1.7 ± .26	— .8 ± .36	>.05
2. " "	4 days	6	12.5 × 3	2.7 ± .42	.8 ± .41	—1.8 ± .42	<.01
3. T + T*	4 "	7	12.5 × 3	2.8 ± .52	7.7 ± .40	+4.9 ± .56	<.001
4. " + 4°C for 15 hr	4 "	7	12.5 × 3	2.8 ± .52	3.9 ± .49	+1.1 ± .55	>.05

* T + T = Thyroidectomized + thyroxine.

occurred(3,4). The thyroid hormone is only one of many factors concerned in regulating the metabolic rate of tissues, and the rate at which the hormone is metabolized probably depends to some extent on the intensity with which other calorigenic influences are acting simultaneously.

For the most part, the PBI concentration in the normal group of animals remained constant unless disturbed by some experimental procedure. At the time of the experiments on the effects of cortisone, however, the initial (*i.e.,* control) values were significantly reduced (Table III, No. 1 and 2). The cause of this reduction is obscure, but whatever the cause it is apparently consistent, since the control value remained steady during the period (Table III, No. 1 vs No. 2 "initial PBI") and experimental variation as reflected by the standard error was no greater at this time than at any other. The constancy of the control values at this time therefore validates the experimental observations in spite of the fact that the actual initial value was at a somewhat unusual level.

It therefore appears that several different mechanisms operate to alter the PBI. Cold stress and exhausting exercise appear to cause an increased rate of removal of the hormone. This effect is somewhat balanced by an increase in the rate of release of thyroid hormone. In direct opposition to this sequence of events is the effect of activating the adrenal cortex. The cortical hormones appear to reduce the rate of disappearance of thyroxine and to reduce the synthesis (though not necessarily the rate of release) (2,17) of the thyroid hormone. As a result, cortisone tends to raise the PBI by decreasing its destruction and to lower the PBI by reducing the synthesis of the thyroid hormone. This antagonism between the peripheral and glandular effects of cortisone may explain the fact that the PBI is not invariably depressed by cortisone in man(3,4,16).

The depression of the plasma PBI observed after 24 to 72 hours of starvation is probably a result of reduced thyroid activity, since it has been shown that the I^{131} uptake is reduced after 48 hours of fasting(8), and that the concentration of thyroid stimulating hormone in the blood is depressed after 4 to 8 days of starvation(15). No attempt was made to determine the effect of fasting on the PBI of the thyroxine-maintained thyroidectomized animal, however.

These experiments do not justify specific conclusions as to the effect of stress or cortisone upon the metabolic fate of thyroxine. Although it seems likely that the alterations of the rate of disappearance of thyroxine from the blood reflect changes in the utilization or catabolism of the hormone, the possible role of increased excretion as a result of alterations in renal or intestinal function cannot be ignored.

Summary. The plasma protein-bound iodine concentration of the intact rat is decreased after prolonged fasting, exhausting exercise and exposure to cold. This effect is not merely a result of suppressed thyroid function, since exercise and cold stress increase the rate of disappearance of injected thyroxine in thyroidectomized rats. Cortisone reduces the rate at which thyroxine is removed from the plasma, and can prevent the increase in the rate of disappearance of the hormone which is normally produced by exposure to a cold environment.

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